9 MAY 1959
READING

JOURNAL OF EXPERIMENTAL BOTANY

AN OFFICIAL ORGAN OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY

Volume 10 *February* 1959 *Number* 28

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OXFORD: AT THE CLARENDON PRESS

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The Physiology of the Sieve Tube

PRELIMINARY EXPERIMENTS USING APHID MOUTH PARTS

P. E. WEATHERLEY, A. J. PEEL, AND G. P. HILL

Department of Botany, University of Nottingham

Received 7 July 1958

SUMMARY

The willow aphid *Tuberolachnus salignus* feeds by inserting its stylets into a single sieve element. If the insect is severed from its proboscis whilst feeding, sap from the sieve tube exudes from the cut end of the stylet canal and may continue for many hours. By attaching a capillary tube the rate of exudation can be measured and the sap analysed. Normally the rate of exudation was about 1 mm.^{3} per h. This means a movement of water and sucrose of 100 cm./h. or 100 sieve elements per min. The sap was found to contain between 5 and 15 per cent. sucrose, up to 0·4 per cent. raffinose, no reducing sugars, and about 0·5 per cent. amino-acids.

Girdles some distance above and below exuding stylets failed to stop exudation (Tables I and II). This indicates a rapid sealing of the cut ends of the sieve tube and a switch-over in source of supply from the leaves to storage cells in the stem. The use of isolated stem segments and even irrigated strips of bark followed. Using these, the following points have been established. (*a*) There is no polarity of movement (Table IV); (*b*) a minimal length of about 16 cm. of stem or 800–1,000 sieve elements are needed to give the full exudation rate (Fig. 5); (*c*) when the D.P.D. on the inside of the bark is raised (Figs. 6, 7, and 8), the rate of exudation declines, but the concentration of the exudate rises and exudation continues, indicating a maintenance of turgor, even in face of a D.P.D. of 20 atm. or more. Secretion of sugar into the sieve tube continues albeit slowly even against a sieve tube concentration of 50 per cent.

These findings are discussed in relation to current theories of translocation and are considered to favour the mass flow hypothesis.

INTRODUCTION

WHILST it is now generally agreed that organic compounds synthesized in leaves are transported throughout the plant via the sieve tubes of the phloem, there is no such unanimity on the mechanism of transport. Diffusion was shown many years ago to be some thousands of times too slow, whilst rapid movement at an interface in the cells has seemed unlikely in view of the variety of compounds transported. The idea put forward by Münch (1930) of mass flow in the sieve tubes in response to differences in turgor pressure has provided a workable hypothesis, its chief obstacle being the structure of the sieve tube with its frequent sieve plates, seemingly barriers to free flow (Hepton *et al.*, 1955). More information bearing on the nature of sieve-tube translocation has been obtained using a technique devised by Kennedy and Mittler (1953) for studying the feeding of aphids. The willow aphid

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Tuberolachnus salignus (Gmelin) feeds by thrusting its stylets into a sieve tube. By severing the proboscis near the head of the insect whilst it is feeding, the stylets may be left undisturbed buried in the bark and form an inert tube leading from the sieve tube to the exterior. Kennedy and Mittler (1953) and Mittler (1957, 1958) found that sap continued to be exuded by the decapitated stylets, presumably due to the turgor pressure in the sieve element, and could continue for 24 hours or more. Sectioning revealed that the stylets penetrated a single sieve element, thus the exudation was the outflow from an individual element. The sap could be collected in a capillary tube held close to the stylets and the rate of exudation and its chemical composition investigated. In this paper preliminary experiments are described in which this technique has been applied to the study of sieve-tube physiology.

MATERIAL AND METHODS

The technique used was essentially similar to that of Kennedy and Mittler (1953) and Mittler (1957, 1958). Several species of willow were used, but principally *Salix viminalis*, the common osier, in the form of young potted trees or cuttings rooted in sand. In winter cuttings could be made to root and produce leaves without difficulty by using bottom heat and artificial light (fluorescent or mercury vapour lights). Colonies of the aphid were readily maintained on the sprouted cuttings. The aphids were placed on the experimental stem and their movement restricted by enclosure in a cellophane or perspex cage until they settled down to feed. The stem was now clamped in a horizontal position and the aphid anaesthetized with a gentle stream of carbon dioxide. Using a binocular microscope and a fine scalpel made from a sliver of razor blade, the proboscis was then severed as close to the head of the insect as possible. The labial sheath surrounding the stylets was a hindrance to the sap collection and was removed with a fine brush. On cutting the proboscis, sap was exuded from its cut end to form a drop. This could be collected by bringing the end of a fine glass capillary tube close enough to make contact with the drop which was drawn, together with subsequent exudate, into the capillary by surface tension. The capillary tube was held by a manipulator permitting movement in three directions at right angles and the volume of exudate was measured as the length of the column of liquid in the tube using a vernier microscope. The capillaries used ranged from 0·5 to 0·1 mm. internal diameter.

Paper chromatography was used for qualitative and rough quantitative analysis of the carbohydrate and amino-acid content of the sap. Since, apart from a little raffinose, sucrose was the sole carbohydrate constituent of the sap, and its amino-acid concentration was usually less than 1 per cent., it was sufficiently accurate, and very convenient, to use a refractometer to measure sucrose concentration. Huber *et al.* (1937) and Tingley (1944) used the refractometer on the same grounds. The refractive index of as little as 0·5 mm.[3] could be measured, enabling readings to be taken at short time intervals.

EXPERIMENTAL RESULTS

Evidence that stylet exudation is relevant to normal translocation

Whilst there is good evidence that the stylets puncture a single sieve element, the possibility cannot be overlooked that the exudate, although persisting for long periods, stems from the cells immediately surrounding the element, normal movement along the sieve tube playing no part in it. The following considerations establish that stylet exudation involves normal translocation.

FIG. 1

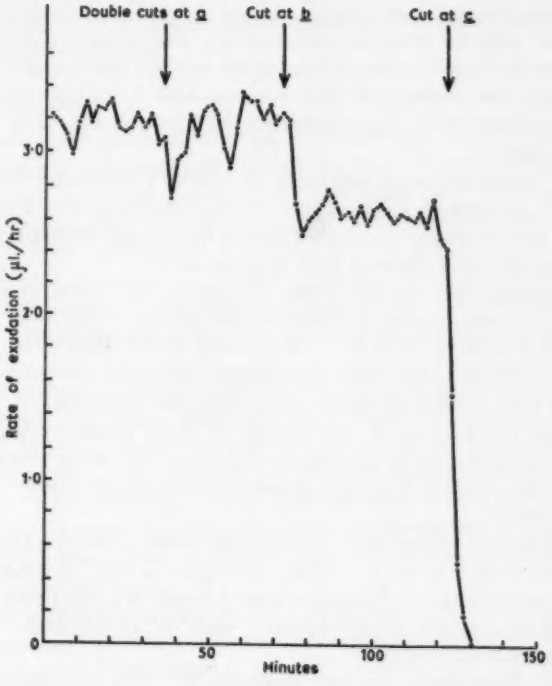

FIG. 2

FIGS. 1 and 2. 1. Ringed stem showing position of incisions in the bark in relation to stylets (for explanation see text). 2. The effect on rate of exudation of the incisions shown in Fig. 1.

Effects of incisions in the bark. A girdle of bark was removed near the base of a stem and an exuding proboscis established about 2 cm. above it as shown in Fig. 1. If now two parallel longitudinal cuts were made as little as 5 mm. away from the proboscis on either side (Fig. 1*a*), the exudation remained unaffected as shown in Fig. 2. But when the bark was cut transversely as much as 6·5 cm. vertically above the stylets (Fig. 1*b*), their rate of exudation

very soon slowed down (see Fig. 2), whilst a cut 2 cm. away (Fig. 1*c*) caused complete stoppage although even here it was four times farther away from the proboscis than the cuts at *a*. In other experiments it was shown that the response to cuts vertically above the stylets as at *b*, was unaffected by the presence or absence of longitudinal cuts at *a* which have been made as close as 3 mm. with no more than a transient effect on the rate of exudation. Cuts closer than 3 mm. disturb the stylets and exudation stops. The effect of transverse cuts farther from the stylets than the 6 or 7 cm. chosen for these experiments will be described on p. 8.

Response to the physiological state of the leaves. Normally the sap contained around 10 per cent. sucrose and 0·5 per cent. amino-acids. If, however, plants were kept for some days in darkness, the leaves turned yellow. At the same time the sugar content of the stylet exudate was found to fall to a mere trace whilst the amino-acid concentration rose to as high as 5 per cent. This is consistent with the well-known fact that in the dark leaves export their nitrogen.

These results parallel those of Mittler (1958) who found that the amino-acid content of stylet exudation from naturally senescing plants increased, whilst the content decreased during bud break. Thus the stylets appear to tap a normal food channel from the leaves.

Quantitative considerations. The rate of stylet exudation gives a measure of the passage of water and sucrose into the particular sieve element punctured. If it is assumed that they are entering by diffusion over the whole surface of this individual element, approximate values for the permeability constants of the sieve element to water and sucrose can be computed, provided its dimensions are known. A comparison of these estimates with the permeability constants normally found for other plant cells will give some indication of the likelihood of water and sucrose arriving from adjacent cells by ordinary diffusion.

A typical willow sieve element was found to be 170 μ long and 23 μ diameter. Assuming it to be a cylinder, this gives a total surface including the sieve plates of 13,109 μ^2. Exudation rates between 0·5 and 3 mm.^{3} per hour occurred. For the purpose of calculation 1 mm.^{3} of 10 per cent. sucrose solution per hour will be assumed.

(i) *Permeability constant for water* (P_w). The difference in D.P.D. between the sieve element and its surrounding cells is unlikely to be more than 5 atm. even if the surrounding cells were fully turgid.

Thus

$$P_w = \frac{10^9}{13109 \times 5 \times 60} = 254\mu/\text{atm./min.}$$

The usual figure for the permeability of plant cells to water is 0·2 μ/atm./min.

(ii) *Permeability constant for sucrose* (P_s). The quantity of sucrose arriving in the sieve element is 0·1 mg. or $0{\cdot}1 \div 342 \times 1000$ gm. mols./h. If the difference in sugar concentration between the sieve tube and the surrounding cells

is taken as 10 per cent. (i.e. surrounding cells at 20 per cent. level) this is 1/3·42 gm. mols. per litre.

$$P_i = \frac{0{\cdot}1}{342 \times 1000} \times \frac{3{\cdot}42}{13109 \times 60}$$

$$= 1{\cdot}3 \times 10^{-12} \text{ gm. mols./}\mu^3\text{/mol. conc. diff./min.}$$

The usual figure for plant cells is between 10^{-17} and 10^{-20}.

Thus, to account for the arrival of water and sugar in the sieve element by normal diffusional processes the sieve element permeabilities would need to be 3 and 5 orders of magnitude greater than normal, respectively. This seems unlikely. A special, more rapid longitudinal movement such as that of normal sieve-tube translocation is indicated.

Experiments with whole plants

The rate of exudation and composition of the sieve-tube sap. The rate was found to vary according to circumstances between 0·5 and 3·0 mm./h. About 1 mm./h. was commonly obtained, which agrees with Mittler's (1957, 1958) finding. Taking the diameter of the sieve tube as 23 μ, this represents a rate of movement at the site of stylet insertion of about 1 m./h. if the sap is considered to converge on the stylets from both directions. This is of the same order as the classical estimate of translocation rate of Dixon and Ball (1922) and at a conservative estimate it means that the sap moves through 100 sieve elements per minute.

Chromatographic analyses have confirmed those of Mittler (1958) on willow and Zimmermann (1957) on *Ulmus americana* and *Fraxinus americana*. The sieve tube sap is a solution of between 5 and 15 per cent. sucrose with up to 0·4 per cent. raffinose and a total of about 0·5 per cent. amino-acids, which have been identified by Mittler (1954, 1958). There seemed to be a complete absence of hexose and hexosephosphate. As mentioned above, the composition of the sap, especially the sucrose : amino-acid ratio, depends on the physiological state of the exporting leaves.

Wilting caused a marked decrease in rate of exudation with an accompanying increase in concentration, but with a normal supply of water to the roots the exudation showed remarkable constancy of rate and concentration. Measurements of sucrose concentration during the day and evening were made in three experiments, shown in Fig. 3. These plants were in bright diffuse light near a window and experienced a decline in light intensity in the evening. In spite of these changes the sucrose concentration in the sieve tube remained unchanged in each experiment though at this stage diurnal changes in response to day and night were not investigated.

Interruption of pathway by incisions. Münch's (1930) incision experiments helped him to formulate his mass-flow theory. He found that exudation from a cut into the bark stopped when he made another cut directly above it, indicating a rapid downward flow. Moose (1938) could not confirm this when

he made similar experiments on a number of tree species. Our experiments also failed to support those of Münch.

A number of experiments were carried out using cuts and girdles both above and below exuding stylets. The cuts were made down to the xylem,

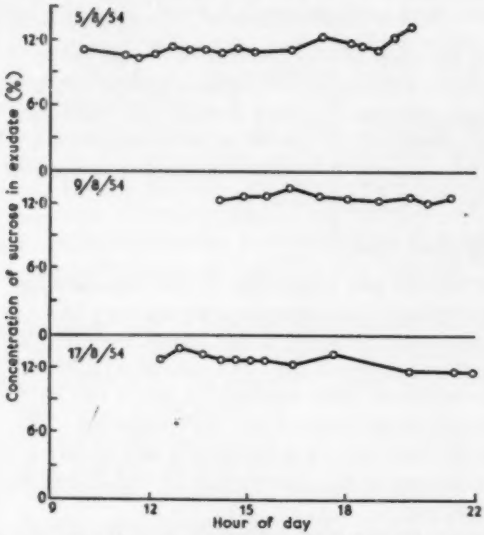

FIG. 3. Variations in sucrose concentration of sieve-tube exudate during the afternoon and evening. Data from three different plants.

1–2 cm. long in a transverse direction. Girdling consisted of removing a ring about 1 cm. wide of all the tissues external to the xylem. The results of two experiments are shown in Table I, in which the sucrose concentration of the exudate was followed before and after girdling. In the first experiment (upper half of Table I) the stem was girdled 55 cm. above exuding stylets. It will be seen that during the ensuing 3 hours there was no apparent effect on the exudation (rates of exudation were not measured but no obvious changes in rate were observed). Four days later exuding stylets were established at 12, 20, and 50 cm. below the girdle. The sucrose concentration had fallen by about one-third, but there was no difference between the three positions. On the following day little further change in concentration had taken place.

In the second experiment (lower half, Table I) the stem was girdled 15 cm. above exuding stylets. This appeared to result in an immediate fall in concentration, but within an hour the concentration recovered to nearly its original value. The following day the stem was girdled near its base, and readings taken subsequently that day were slightly lower but those taken 3 and 4 days later showed little further change in concentration. Readings

TABLE I

Sucrose concentration (per cent.) of the exudate before and after girdling above and below the stylets, and a comparison of the final concentrations above and between girdles

Date	Time of day	Position of stylets in cm. below girdle	Sucrose conc.
10.6.54	12.00–12.30 p.m.	—	9·8
	12.30–1.00 p.m.	—	9·2
	Stem girdled at 2.10 p.m.		
	2.15–2.55 p.m.	55	8·4
	2.55–3.35 p.m.	55	9·0
	3.35–4.25 p.m.	55	10·4
	4.25–5.15 p.m.	55	9·9
14.6.54	9.55–10.45 a.m.	12	6·9
	12.05–12.30 p.m.	20	7·2
	2.25–3.10 p.m.	50	7·2
15.6.54	3.30–3.50 p.m.	50	7·0
	3.50–4.10 p.m.	50	7·0

Date	Time of day	Sucrose conc.
17.6.54	9.30–9.50 a.m.	16·8
	9.50–10.10 a.m.	15·8
	10.10–11.10 a.m.	15·1
	Stem girdled 15 cm. above stylets	
	3.00–3.30 p.m.	10·1
	3.30–4.20 p.m.	14·1
18.6.54	11.15 a.m. Stem girdled near base	
	11.30 a.m.–12.15 p.m.	10·0
	12.15–2.15 p.m.	12·5
	4.00–4.45 p.m.	12·5
21.6.54	11.00 a.m.–12.15 p.m.	10·7
	12.15–1.50 p.m.	13·5
22.6.54	9.30–11.00 a.m.	10·2
	11.00–11.45 a.m.	8·2
	11.45 a.m.–1.00 p.m.	11·0
	1.00–2.30 p.m.	10·2
	Readings below are from just above girdle	
	2.30–2.50 p.m.	14·4
	2.50–3.30 p.m.	17·1
	3.30–4.25 p.m.	15·1
	4.25–4.50 p.m.	15·9

taken on the last day immediately above the upper girdle showed the concentration there to be equal to the original value before girdling.

The striking feature of these results is not so much the slight decline in concentration below a girdle but that the concentration was maintained at so high a level and for so long even from a segment isolated by girdles above and below. This has two important implications. Firstly, if there is a mass flow under pressure in the sieve tubes, then the failure of an incision to stop this

flow implies a self-sealing mechanism preventing any considerable leakage from the cut and allowing a pressure to be built up within the sieve tube on either side of the cut. Secondly, if the normal source of supply of organic solutes is the leaves, there must be a switch over to another source, presumably storage cells in the stem itself, when the supply from the leaves is cut off by girdling. Furthermore, this maintained exudation from between girdles suggested the use of isolated segments as a convenient technique for studying certain aspects of sieve-tube physiology.

Experiments with isolated segments

Mittler's (1957) finding that aphids will continue to feed on isolated segments for only 2 to 3 hours and eventually disperse was confirmed. That this is due to an adverse water balance causing a drop in turgidity was shown by supplying the stems with water, when exudation from stylets or the feeding of aphids continued indefinitely.

The stem segments were prepared by cutting approximately 20 cm. lengths of branches about 2 cm. in diameter. No special precautions were taken to prevent air from entering the xylem, but glass tubes were connected without delay to either end of the segment by means of rubber tubing and these were filled with water and held upright to act as reservoirs.

Stylets embedded in isolated segments continued to exude for many hours with only slow diminution in concentration, as shown in Table II. It will be seen that the concentration declined by about a third in the course of 12 days. A further development of this technique was the use of isolated strips of bark. A window 10 cm. long by 2 cm. wide was cut in the side of a polythene tube 2 cm. in diameter and open at both ends. A strip of bark slightly larger than the window was fixed at its edges over the window with adhesive tape which was subsequently waxed to give a watertight seal. Aphids confined on such strips would feed, though somewhat more reluctantly, and on severing the stylets normal exudation occurred. The advantage of this technique was that the inner surface of the bark could be readily irrigated by any solution filling the tube.

No polarity in sieve-tube translocation. This was demonstrated in the following way. Aphids were confined to the opposite ends of a stem segment. First a proboscis was cut near, say, the morphologically lower end, and the rate and concentration of the exudation measured. Here the sap was coming to the stylets principally from above. Then stylets were cut near the morphologically upper end of the segment and the rate and concentration again measured, the sap this time coming from below. Comparison of rates and concentrations of exudate obtained from either end of a number of segments is shown in Table III. No significant difference was found.

The minimal functional length of a sieve tube. The exudation from an isolated segment clearly shows that sieve tubes the length of the segment will function. The question arises: what is the minimum length that is functional? To investigate this a proboscis was sited about 2 cm. above a girdle near the basal

TABLE II

Changes in sucrose concentration of exudate from an isolated stem segment

Date	Time of day	Sucrose conc. in %
18.8.54	10.05–10.30 a.m.	6·1
	10.30–11.15 a.m.	5·8
	11.15 a.m.–12.15 p.m.	6·0
	12.15–1.20 p.m.	6·0
	1.20–3.25 p.m.	6·0
23.8.54	9.55–10.25 a.m.	5·5
	10.25–11.10 a.m.	5·9
	11.10–11.40 a.m.	5·4
30.8.54	9.45–10.20 a.m.	3·9
	10.20–10.50 a.m.	3·9
	11.25–11.55 a.m.	4·3

TABLE III

Comparison of rate of exudation and concentration of exudate from stylets sited near the apical and basal ends of isolated segments of willow stem

	Mean rate of exudation in μ/h.			
Segment	Apical	Basal	Difference	Least significant difference
a	1·21	1·19	0·02	0·82
b	1·74	1·99	0·25	0·77
	Sucrose Conc. (%)			
Segment	Apical	Basal	Difference	Least significant difference
a	13·0	12·6	0·4	3·3
b	13·5	13·3	0·2	4·1

end of a segment 30 or 40 cm. long. The rate of exudation and concentration of exudate were then measured continuously whilst a series of transverse razor cuts through the bark was made, starting some 20 cm. or more above the stylets and approaching them by about 2 cm. at each cut. After each cut an interval was allowed for any effect on the exudation to be manifest.

The results of such an experiment are shown in Fig. 4. The first cuts, made 18 cm. away, had no apparent effect. However, when cuts were made 10 cm. from the stylets (i.e. 11·5 cm. from the girdle) or less there was an immediate slowing in rate of exudation followed by partial recovery to reach a lower steady rate. With these lower rates the concentration of sucrose tended to be lower. The relationship between rate of exudation and total length of sieve tube functioning (i.e. the distance between the girdle and the cut in each case) is shown in Fig. 5. Curve 'a' refers to the above experiment whilst 'b' and 'c' represent two further experiments. These are typical of the results of a number of experiments the results of which were very consistent and showed clearly that approximately 16 cm. of sieve tube of 800–1,000 sieve elements are necessary for the full exudation rate. Decreasing this length caused

progressive lowering in rate until a cut leaving a 3 or 4 cm. length of bark stopped exudation completely. This does not necessarily mean that a certain minimal length of sieve tube is required for it to function. In one experiment

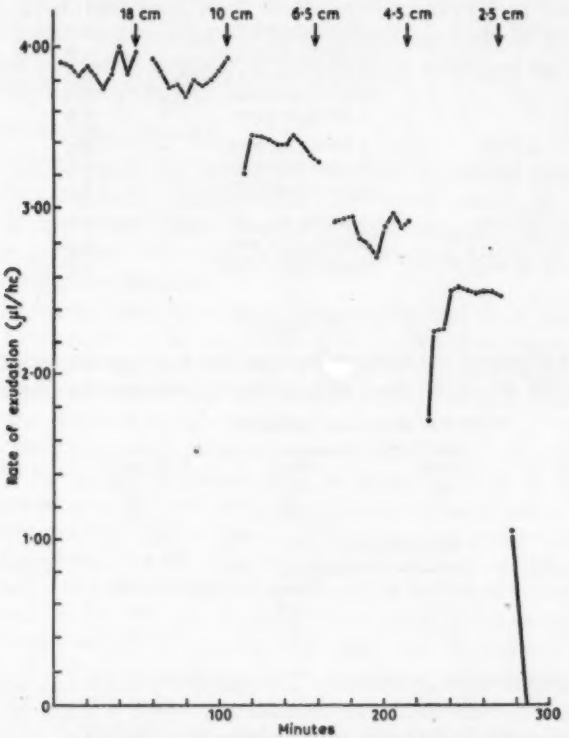

FIG. 4. The effect on rate of exudation of incisions in the bark made progressively nearer to the stylets.

quite a rapid exudation was maintained from a 3 cm. length of bark which had been isolated by girdles prior to cutting the proboscis (Fig. 5, point P). It is likely that the stoppage caused by close cuts was due to mechanical disturbances in the act of cutting.

Osmotic effects. The movement of water into the sieve tube to make good that lost through the stylets presumably comes from surrounding cells. Its influx will therefore depend on the difference in D.P.D. between the sieve elements and these adjacent cells. In an isolated segment with water-filled xylem, this water-supply will be maximal being at zero D.P.D. The effect of altering the D.P.D. of the water-supply was investigated by running various osmotica through the xylem. Solutions of sucrose or mannitol were forced under pressure into the cut end of the segment and the concentration of the

fluid issuing from the other end measured at intervals. At the same time the rate and concentration of the stylet exudate were followed.

The results of two such experiments are shown in Figs. 6 and 7. In the

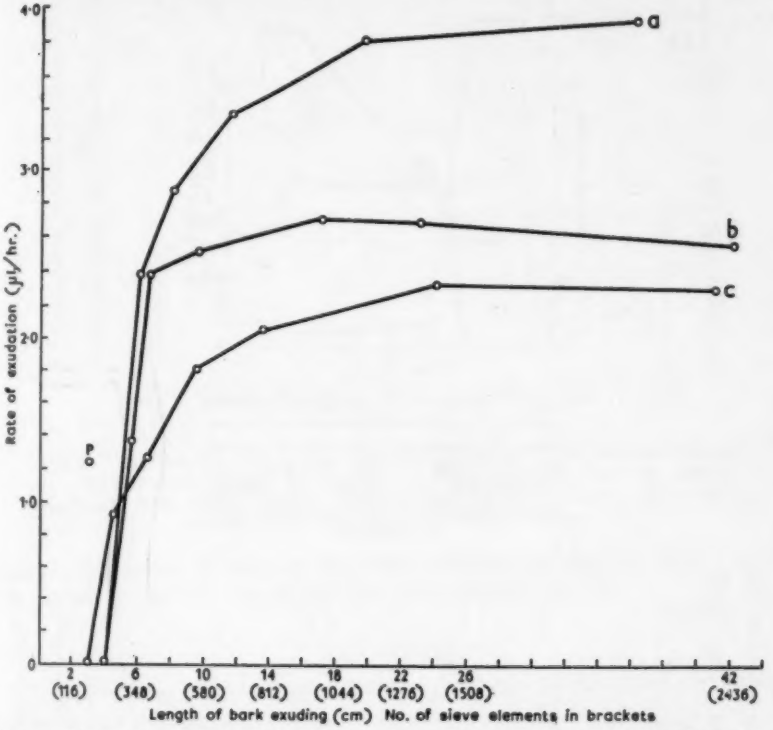

FIG. 5. Curves showing the relationship between rate of exudation and the length of sieve tube functioning. Data from three experiments.

first 25 per cent. sucrose solution and in the second 15 per cent. mannitol solution was forced through the xylem. It will be seen that as the concentration in the xylem increased, the rate of exudation was reduced and reached a very low value. At the same time the concentration of the exudate increased, finally approaching a sucrose concentration of 50 per cent. A figure proportional to the rate of secretion of sucrose into the sieve tube can be obtained as the product of exudation rate and concentration: such rates are shown in parenthesis adjacent to the concentration curve. Clearly the secretion was greatly reduced when the D.P.D. in the surrounding tissues was raised to between 20 and 30 atm. But it is notable that exudation proceeded even under these conditions, indicating the maintenance of a positive pressure in the sieve tube. In the second experiment the mannitol solution was finally replaced by water, and it is clear that the changes were entirely reversible.

Changing the solution in the xylem was rather slow. The effect of more immediate changes was investigated using the bark-strip technique. The results of a single experiment are shown in Fig. 8. They confirm the previous

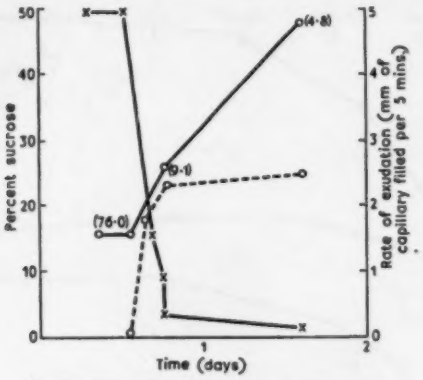

FIG. 6. The effect of passing 25% sucrose solution through the xylem vessels. Concentration of sucrose in xylem effluent (o---o). Rate of exudation from stylets (x——x). Concentration of sucrose in exudate (o——o). Figures for sucrose secretion given in brackets.

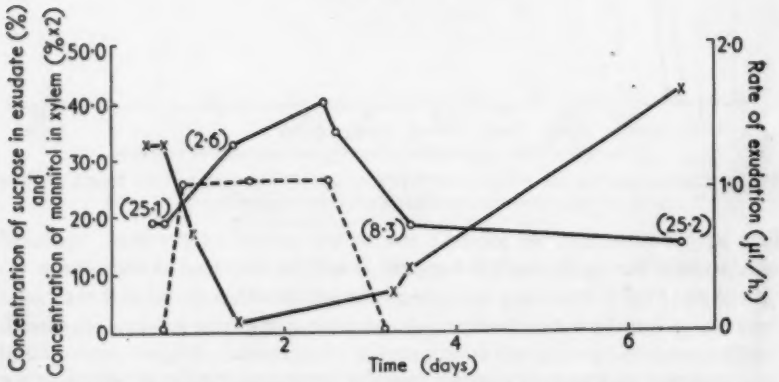

FIG. 7. The effect of passing 15% mannitol solution through the xylem vessels and later replacement by water. Concentration of mannitol in xylem effluent (o---o). Rate of exudation from stylets (x——x). Concentration of sucrose in exudate (o——o). Figures for sucrose secretion given in brackets.

experiments and in addition show a much more rapid response in rate of exudation followed by a somewhat slower response in concentration of exudate. Once again, when the osmotic potential of the bathing solution was

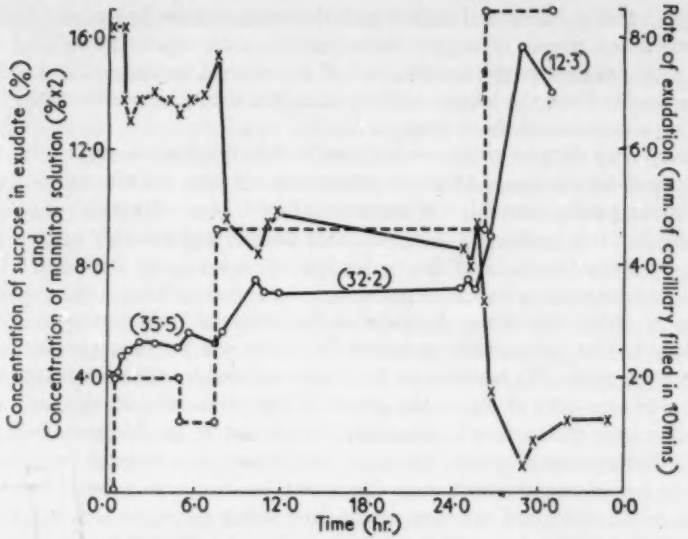

FIG. 8. The effect of irrigating the inner surface of the bark with mannitol solutions of various concentrations. Concentration of mannitol solution (o---o). Rate of exudation from stylets (x—x). Concentration of sucrose in exudate (o—o). Figures for sucrose secretion given in brackets.

raised, the decline in rate was proportionately greater than the rise in concentration so that the rate of sugar secretion appeared to fall.

DISCUSSION

Considerations of the composition and high rate of the stylet exudation show that its constituent water and sugar are unlikely to pass into the individual pierced sieve element by diffusion over its surface. Some special form of movement is indicated. The incision experiments showed the movement to be longitudinal, i.e. along the sieve tube, and this, together with the dependence of the composition of the sap on the physiological state of the leaves and the similarity of the rate to accepted estimates for normal translocation, makes it likely that the stylet exudation is a genuine sample of the translocation stream. Mittler (1958) came to the same conclusion.

Exudation from isolated stem segments suggests that it is the individual sieve element with its surrounding cells which is the functional unit and not the sieve tube as a whole. Clearly the leaf mesophyll plays no special part, but the adjacent cells anywhere along the sieve tube may act as a source or a sink for transportable materials, according to circumstance. This explains the failure of attempts to repeat Münch's incision experiment. In our experience an incision made some distance above an exuding proboscis may cause a transitory slowing or stoppage, but it appears that in willow, at least, the

incision rapidly becomes blocked and the storage cells below the incision become a new source of supply and exudation soon regains its original rate. This may also explain the seeming lack of any diurnal variations in exudation, for as supply from the leaves declined at night, the storage cells of the stem may have maintained the supply.

Clearly two distinct processes are involved in the functioning of the sieve tube. First, the exchange of water and solutes between the sieve tube and its surrounding cells. Second, the movement of the same along the sieve tube. For the first it is tentatively suggested that sucrose and possibly other solutes move by active transfer and that water follows passively by diffusion. This is consistent with the apparent permeability data taken in conjunction with the figure of about 800 sieve elements as the minimal unit giving maximum exudation. The permeability constant for water was between 500 and 1,000 times too great on the assumption that water entered by diffusion through the surface of one sieve element. But clearly if 800 elements are taken as a unit, diffusion over the surface is adequate. This is not so in the case of sucrose, with a discrepancy of several thousand, and here active transfer is indicated.

With regard to movement along the sieve tube, clearly there must be a mass flow into the stylet end and thus a mass flow within the vacuole of the pierced sieve element. The data presented here give no direct evidence of mass flow occurring across the sieve plate. But they do show that *water* moves along the sieve tube at the rate of at least 100 sieve elements per minute, that its movement is non-polar, and that its rate declines if the turgor of the cells is reduced by a plasmolyticum. These facts are consistent with mass flow and inconsistent with diffusional and surface-movement hypotheses in which the solutes move and the water remains static. The chief obstacle to the mass-flow hypothesis is the structure of the sieve tube. Currier, Esau, and Cheadle (1955) have recently shown the sieve element to be a living plasmolysable cell; this is not inconsistent with a mass flow in the vacuole, but the lack of continuity of the vacuoles through the sieve plates (Hepton *et al.*, 1955) would seem to preclude mass flow through them. Yet so sensitive are the living sieve elements to manipulative techniques that evidence of complete blockage of the sieve plates from cytological preparations cannot be regarded as conclusive.

Certainly mass flow is feasible were the sieve areas open. If the radius of the willow sieve tube is taken as $11{\cdot}5\,\mu$ and there are 60 sieve plates per cm. each being $5\,\mu$ thick and possessing 5 sieve areas which together make up half the total cross-sectional area of the sieve tube, application of the Poiseuille equation indicates that a pressure gradient of only about 1 atm./m. would be needed to produce normal translocation rates. Even if the sieve plates appear to be filled with cytoplasm, this may well have special properties allowing mass flow as envisaged by Crafts (1948). Mittler has also applied the Poiseuille equation to the flow through the stylet canal and obtained a figure of 10·20 atm. for the turgor pressure of the sieve tube. Our own measurements have given a similar figure. Thus it is quite evident that the sieve element is capable of

generating the considerable turgor pressure which would be needed for mass flow.

What is the relationship between the two constituent processes discussed above and how is translocation regulated? The results suggest very tentative answers to these questions. In an isolated segment uniform conditions prevail and no movement occurs in the sieve tube which would be in a state of uniform turgor. When punctured the vacuolar contents of the sieve element are extruded through the stylets. This results in a fall in turgor pressure and hence a mass flow along the sieve tube towards the stylets. In addition the fall in turgor will be accompanied by a rise in D.P.D. which will lead to an influx of water by diffusion from surrounding cells and thus to a dilution of the vacuolar contents of the sieve tube. It is suggested that it is this dilution which initiates sugar secretion from surrounding cells, sucrose transfer being a function of the sucrose concentration in the sieve tube. The osmotic experiments provide evidence for this: as shown above (p. 11) the secretion was depressed by increasing sieve tube concentration.

More precisely it is envisaged that movement both into and out of the sieve tube is in response to difference of 'sucrose potential'. This is related to, but not simply equated with, differences in *concentration* since transfer is presumed to be 'active' and could occur against a concentration gradient. Regions of sucrose utilization having a low 'sucrose potential' will act as 'sinks' and sucrose will move into them out of the sieve tube. Regions of synthesis or mobilization of reserves will have a high 'sucrose potential' and sucrose will be secreted into the sieve tube. Thus the sucrose in the sieve tube is in dynamic equilibrium with that in the surrounding cells, any of which can act as 'source' or 'sink' according to the direction of the potential gradient.

In whole plants during photosynthesis the 'sucrose potential' in the mesophyll is higher than in the sieve tubes into which it moves and therefore initiates a mass flow. The translocation stream in its passage down the stem will lose sucrose whenever the surrounding cells are of lower 'sucrose potential'. Again, it is the 'sucrose potential' which controls the switch-over in source of supply following girdling which was discussed above.

This view, which is based essentially on that of Münch and Crafts, maintains that the motive power of translocation is the secretion of solutes into and out of the sieve tube. The major solute is sucrose, but any substance finding its way into the sieve tube will be carried in the stream, and under certain circumstances other solutes may provide the motive power as in senescing leaves where the amino-acid content may achieve considerable proportions.

It is evident from the results of the osmotic experiments that cells surrounding the sieve tubes may develop a very considerable 'sucrose potential', for secretion into the sieve tube continued when its vacuolar contents contained as much as 50 per cent. sucrose. This is undoubtedly of major significance to the functioning of the sieve-tube system, for it means that the high turgor pressures essential to mass flow can be maintained in the sieve tubes in the face of considerable water stress in the plant.

ACKNOWLEDGEMENTS

Thanks are due to the Agricultural Research Council and the University of Nottingham for research grants received by two of us (A. J. P. and G. P. H. respectively). Some of the results contained in this paper formed part of a thesis submitted by one of us (G. P. H.) for the Degree of M.Sc. in the University of Nottingham.

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The Use of C^{14}-Proline by Growing Cells: Its Conversion to Protein and to Hydroxyproline

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Received 5 March 1958

SUMMARY

C^{14}-proline is readily absorbed by growing tissue cultures of carrot root phloem and of potato tuber in experiments carried out under aseptic conditions. The C^{14}-proline rapidly enters into the protein of the tissue, appearing there in as short a period as 15 minutes, and, thereafter, the amount incorporated into the protein bears a linear relation to time. Virtually all the C^{14} appears in the protein hydrolysate in the form of proline and hydroxyproline. It is shown that the conversion from proline to hydroxyproline occurs *after* the C^{14}-proline is combined into the protein and that this conversion proceeds progressively with time. The ratio of C^{14} as proline to C^{14} as hydroxyproline declined progressively from a value of 4·0 after 30 minutes of contact and seemed to become stabilized eventually at 0·7. C^{14}-hydroxyproline, which can be absorbed by the tissue, seems *not* to be incorporated into the protein as such. The protein moiety which contains the C^{14}-hydroxyproline from C^{14}-proline represents a stable protein which is not metabolized and whose carbon does not 'turn over'. This inert protein seems to be characteristic of cells which are in rapid division under the influence of coconut milk or are synthesizing protein in response to other stimuli such as the events at a cut tissue surface. The protein in question seems to be present mainly in the cytoplasm rather than in its particulate inclusions. These results are compatible with earlier views which require that part of the protein in the cell 'turns over' its carbon, whereas another part does not do so.

In cells, growth and protein synthesis proceed concomitantly. Both cell division and cell enlargement are associated with protein synthesis. In recent work in this laboratory, cells stimulated to proliferate rapidly were shown to contain unusually large amounts of hydroxyproline in their alcohol-insoluble material. C^{14} somewhat unexpectedly entered the protein of actively metabolizing cells more readily if supplied as glucose than if given as glutamine or its derived glutamic acid. On the other hand respiratory $C^{14}O_2$ arose more readily from C^{14}-glutamine or γ-aminobutyric acid than from glucose (Steward, Bidwell, and Yemm, 1956).

These results are understandable if the metabolism of these growing cells is considered in terms of discrete phases or compartments in which a single substrate may participate in one or more reactions, and if some of the carbon from sugar passes through protein synthesis and breakdown prior to its evolution as carbon dioxide.

The role of proline in the cell seemed to be quite distinct from that of glutamine, glutamic acid, or γ-aminobutyric acid. Receiving its C^{14} label from one of the substrates applied, the specific activity of the proline as it

occurred in the protein seemed to be the same as that of the free proline in the cell; and, when the overall growth in fresh weight was accentuated by the coconut milk, both the total protein and the amount of proline and hydroxyproline built into the protein were increased in the same ratio. Therefore, the carbon of proline and of hydroxyproline could *not* participate in the 'turn-over' which characterized the carbon of those protein amino-acids which had received their C^{14} label more directly from sugar.

The explanation advanced to account for these results was that the cells synthesized two metabolically distinct classes of protein. In one case the protein as synthesized 'turned over' and in doing so led ultimately to the release of $C^{14}O_2$. In the biosynthesis of this protein, C^{14}-sugar was the preferred starting-point. The other class of protein was characterized by its failure to 'turn over', and the distinguishing feature was that this protein seemed to incorporate C^{14}-proline *directly* from the cell; and, though C^{14}-hydroxyproline did not occur appreciably in the cell in the free state, it was also contained in this apparently inert protein fraction. While the data were interpreted in terms of two distinctive *kinds* of protein, largely because of the unique feature of the enhanced hydroxyproline content, the behaviour of this apparently non-metabolizable protein could also be due, wholly or in part, to its *location in the cell* as well as to its *composition*. Indeed it is also possible that the protein of 'turn-over' is a particular part (e.g. sidechains or strategically placed amino-acids) of a protein complex of which another part (e.g. a central backbone or inner core) could be regarded as inert. Thus the hypotheses of compartments could be extended to explain the behaviour of the protein as well as of the soluble compounds in the cell. In the case of the protein, however, the possibility also exists that the 'compartments' might not be cytological entities which separate distinct protein molecules, but they may nevertheless exist effectively as different parts of a protein complex, which is not equally accessible to metabolism throughout its entire structure. These considerations, therefore, give added interest to the further studies, which are now to be described, on the behaviour of proline and hydroxyproline in the protein of these cells.

The hypothesis that proline is incorporated directly into protein received support in the following way. When supplied exogenously to the tissue, hydroxy-L-proline did not promote growth, as its characteristic presence in the protein of the growing cells might have led one to suppose, but it proved to be a quite powerful *inhibitor* of growth. Moreover, proline, alone among the amino-acids tested, competitively reversed this inhibition. Thus, when present *free* in the cell, hydroxyproline competes with proline and its inhibitory action on growth and on protein synthesis can be explained. Indeed, certain chemical derivatives of hydroxy-L-proline also act as proline antagonists in a way that becomes intelligible on this hypothesis (Steward, Pollard, Witkop, and Patchett, 1958).

The present paper furnishes further evidence on certain important features of these interpretations, namely the *direct* utilization of C^{14}-proline by the

cell, its incorporation into the alcohol-insoluble nitrogen of the cell and its failure to undergo any further metabolism *except the conversion to hydroxyproline while so incorporated in the protein.* At the same time many of the underlying hypotheses on which the earlier general metabolic scheme was erected (Steward, Bidwell, and Yemm, 1956) received experimental support.

Hydroxy-L-proline is found in the hydrolysates of a rather limited number of proteins of plants and animals and only rarely does it occur free in nature. In the animal kingdom the conspicuous and possibly only occurrence of hydroxyproline is in collagen, in which it always appears although in amounts which vary with the source (Gustafson, 1955; Leach, 1957). In plants, free hydroxyproline is very rare (Steward, Zacharius, and Pollard, 1955), although it frequently occurs in the protein in very small amounts. Allo-hydroxy-L-proline occurs free and in large amounts in sandalwood (*Santalum*) (Radhakrishnan and Giri, 1954). Other reports of free hydroxyproline in plants are based on chromatographic evidence which did not distinguish between the allo and the normal form. As reported previously from this laboratory by Steward, Thompson, and Pollard (1958); and in other short communications published only in abstract form (Steward and Pollard, 1956) it has been shown that hydroxyproline occurs in unexpected amount in the alcohol insoluble nitrogen fraction of tissue cultures derived from the carrot root and from the potato tuber, and it also occurs in crown-gall tumors on *Kalanchöe* and in the tumours that form spontaneously on certain hybrids of *Nicotiana.* On the contrary, hydroxyproline was absent from, or only present in much smaller amount in, the normal tissues from which the tissue cultures or the tumours were derived.

With all these ideas in mind a study of the proline-hydroxyproline metabolism in plants was made, using the carrot-tissue culture system which has been described previously (Steward, Caplin, and Millar, 1952), and which was also used in the study of the respiration and protein metabolism recently reported (Steward, Bidwell, and Yemm, 1956). The main conclusions were noted in a brief abstract (Steward and Pollard, 1957).

EXPERIMENTAL METHODS

The carrot-tissue system permits selected nutrients (in this case C^{14}-proline) to be supplied under aseptic conditions to rapidly dividing cells.

Uniformly labelled L-proline, with a specific activity of 10 μc./μmol., was added to the external solution. The carbon dioxide produced during the experiment was absorbed in dilute alkali, and its radioactivity was determined. C^{14}-hydroxyproline for use as a substrate was obtained biosynthetically from C^{14}-proline as described below. Analytical methods have been described earlier (Thompson and Steward, 1951). In this study attention could be confined almost entirely to the hydrolysate of the alcohol-insoluble fraction, since the substrates in question, namely proline and hydroxyproline, only occur free in the cell in very small amount. The alcohol-insoluble residue was hydrolysed with 6N HCl at 120° for 18 hours, and the excess HCl

was removed by evaporating the hydrolysate to dryness. Hereafter this alcohol-insoluble nitrogen fraction will be referred to in general as 'protein'.

Admittedly the alcohol-insolubility of this material does not alone establish its nature as protein, but the following additional criteria have been applied to that portion of the insoluble residue which contains the hydroxyproline. The hydroxyproline-containing substance cannot be removed from the alcohol-insoluble residue by exhaustive dialysis, or even by electrodialysis, as carried out in the laboratory of Dr. B. Witkop. When tissue extracts are freed of debris and particulate matter by ultracentrifugation (100,000 *g.* for 2 hours), they still contain an undialysable material which on hydrolysis yields hydroxyproline together with all of the other amino-acids expected in a protein hydrolysate. This material is also precipitable by trichloracetic acid.

Two-directional chromatograms were made as required on the alcohol-soluble extract, or on the hydrolysate of the alcohol-insoluble residue from the tissue, using a phenol/butanol system. Radioautographs were made from the chromatograms using no-screen X-ray film supplied by the Ansco Corporation of Binghamton, New York, in 17×21 inch sheets. This extra large film permitted the radioautography of a compiete chromatogram to be made, using only one piece of film.

The experimental design was essentially as follows. Carrot phloem explants originally weighing about 2·5 mg. were allowed to grow for 10 or 11 days in the full nutrient medium, which contains coconut milk, until they had reached the linear phase of the growth curve as this has been previously reported. At this point the tissue explants were transferred aseptically to fresh nutrient medium which contained the radioactive substrate. Thereafter growth was allowed to proceed for varying lengths of time as described below.

The derivation of C^{14}-hydroxyproline from C^{14}-proline in carrot- and potato-tissue cultures

Uniformly labelled C^{14}-proline was added to the external solution in which carrot-tissue explants had been grown for the pre-period of 10 days. After a further period of 72 hours the tissue explants were harvested and analysed by the methods already described. The results are recorded in Fig. 1A, B, C, D.

After 72 hours of contact with C^{14}-labelled proline almost all of the radioactivity in the tissue appeared in the protein (Fig. 1D). Neither proline nor hydroxyproline were detected with ninhydrin on the chromatograms of the alcohol-soluble nitrogen fraction of the tissue (Fig. 1A), although it is true that the radioautograph from this chromatogram, after long exposure, did show a spot which could conceivably be due to C^{14}-proline, but no radioactivity was found in the position of hydroxyproline (Fig. 1B).

When treated with ninhydrin the chromatogram of the protein hydrolysate showed small, but definite, amounts of both proline and hydroxyproline (along with the other protein amino-acids) (Fig. 1C). This was to be expected. However, the radioautograph from this chromatogram showed clearly that

almost all of the radioactivity which had been absorbed by the cell in the form of C^{14}-proline and incorporated into the protein was contained in proline and hydroxyproline. Although the chromatograms were exposed to the X-ray

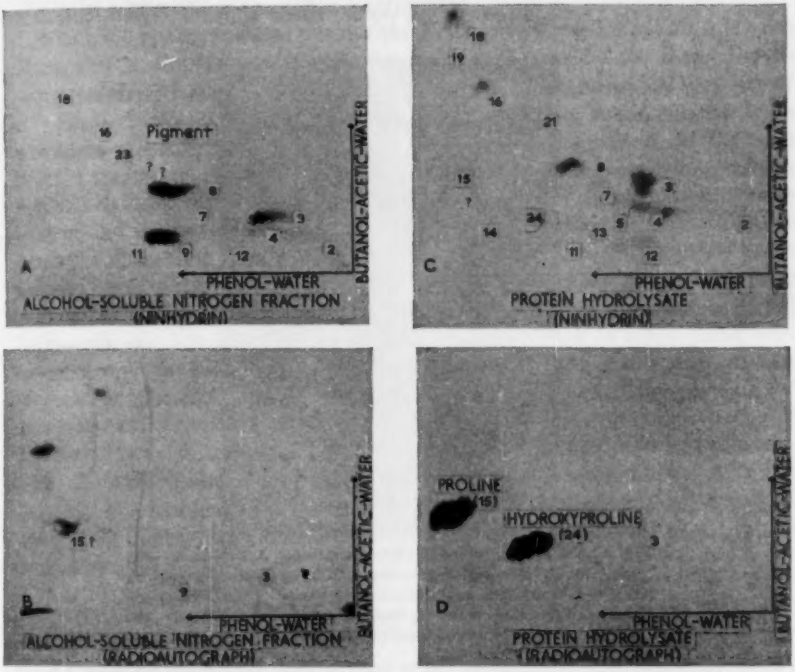

FIG. 1. The metabolism of C^{14} proline shown by chromatograms from cultured carrot explants.

film for as long as 3 weeks, producing thereby very strong spots for proline and hydroxyproline, only very faint and barely detectable spots were formed by the other amino-acids, notably glutamic and aspartic acid. Therefore, these results, illustrated in Fig. 1, establish the fact that hydroxyproline derives from proline, and they also show that these two amino-acids are not appreciably metabolized after incorporation into the protein during 3 days of rapid growth. This distribution of C^{14} in the amino-acids of the protein shows that C^{14}-proline is incorporated directly into the protein; that the conversion to hydroxyproline may then proceed in the protein is demonstrated below.

A similar kind of experiment was carried out using tissue cultures from the potato tuber. These cultures had been established by the method of Steward and Caplin (1951) in media containing both coconut milk and 2:4-D (dichlorophenoxyacetic acid) and, for the absorption period of 72 hours this medium was supplemented by C^{14}-labelled proline. At the end of this

period the tissue was harvested and the alcohol-insoluble fraction was analysed.

The radioautograph illustrated in Fig. 2A shows that the distribution of C^{14} in the protein amino-acids of potato-tissue cultures is qualitatively similar to that of carrot tissue, although the potato tissue appears to have converted

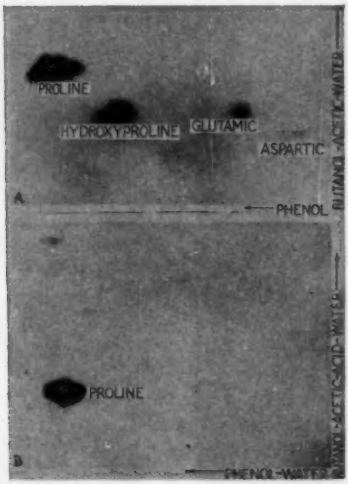

FIG. 2. Radioautographs of chromatograms. A. Of the hydrolysed protein fraction of growing potato explants exposed 72 hours to C^{14}-proline. B. Illustrating the purity of the C^{14}-proline supplies to growing carrot explants. The proline is entirely free of any hydroxyproline.

somewhat more of the proline to glutamic and aspartic acids. Apparently the mechanism of incorporation of proline into protein and its subsequent conversion to hydroxyproline is the same whether the tissue cultures were derived from potato tubers or from carrot roots.

At this point the purity of the C^{14}-labelled proline should be emphasized in order to prove that the hydroxyproline in the tissue could not have been derived from this substance occurring as a contaminant in the proline as supplied.

The radioautograph of a chromatogram of the proline used as a source of C^{14} is shown in Fig. 2B. While this proline contained very minute traces of unidentified substances, it was entirely free of hydroxyproline. The hydroxyproline found in the tissue proteins was not supplied as a contaminant in the proline.

The inert nature of the protein which contains C^{14}-proline and C^{14}-hydroxyproline

The inert nature of the protein fraction that has incorporated C^{14}-labelled proline and C^{14}-labelled hydroxyproline was tested as follows. One hundred

carrot explants were pre-grown in full nutrient medium containing coconut milk for a period of 10 days. At this point 50 uniform explants were selected and transferred to a nutrient medium which contained 25 μc. of uniformly labelled proline, and in this solution they were allowed to grow for a further period of 3 days. Thus the tissue incorporated C^{14}-labelled proline from which C^{14}-labelled hydroxyproline was derived in the manner which has already been illustrated in Fig. 1. At the end of the absorption period the living tissues were transferred aseptically to complete nutrient medium which was free of C^{14}-labelled substrates, and in doing this, batches of 5 tissue cultures were placed in separate culture tubes where their growth continued. At the time of this transfer 2 batches, each consisting of 5 explants, were sampled for analysis to show the status of the C^{14}-labelled compounds in the tissue at the end of the absorption period, but before the subsequent period of further growth. Thereafter samples were taken every 24 hours, each sample again consisting of the 5 cultures contained in a single culture tube, until, after 8 days, all the explants had been harvested. The samples which were collected on the 7th and 8th days of this experiment were grown in tubes fitted with attachments for collecting carbon dioxide, and during the 8 days of this experiment the carbon dioxide evolved was collected periodically and its C^{14} content was determined. After the usual analytical procedures the change in the distribution of C^{14} in the protein amino-acids after 8 days was determined, with the results shown in Fig. 3A and B. It is quite apparent that no other labelled C^{14} amino-acids (other than proline and hydroxyproline) appeared even after a subsequent period of metabolism during which the tissue tripled its weight.

The progressive conversion of proline to hydroxyproline

To determine whether the conversion of proline to hydroxyproline occurred at the instant of its incorporation into the alcohol insoluble nitrogen fraction, or whether it occurred progressively thereafter, a technique was devised for comparing the amount of radioactivity present in the protein as proline or hydroxyproline in the samples collected as described above. Since these two substances contained virtually all the radioactivity in the tissue as seen in Fig. 3, this could be done by the use of one-directional chromatograms in butanol-acetic acid which gave very discrete circular spots. Once separated, the amount of radioactivity present in the form of proline and hydroxyproline was determined directly on the paper, using a tube of the type designed by Fuller (1956), using Geiger gas and a Tracerlab autoscaler.

The ratio of the radioactivity present in the form of proline to the radioactivity present in the form of hydroxyproline was then calculated, and the results are illustrated in Fig. 4A. The figure shows that at the end of the absorption period of 3 days the ratio of C^{14} as proline to C^{14} as hydroxyproline was 1·06, whereas at the end of a further period of 7 days, during which the tissue was metabolizing in a C^{14}-free medium, the ratio fell to about 0·70.

During this period of 7 days the fresh weight of 5 tissue explants increased from 98 mg. to nearly 300 mg. (see Fig. 4B).

It is important to note that the decline in the ratio of proline to hydroxyproline was not due to the loss of C^{14} from the former as CO_2, since the tissue only respired a small amount of the total C^{14} which was absorbed as proline.

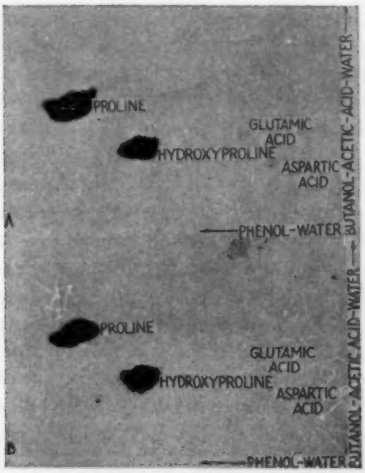

FIG. 3. Radioautographs of chromatograms of the hydrolysed protein fraction of growing carrot explants showing that the C^{14}-proline and C^{14}-hydroxyproline in the protein are not metabolized further. A. Tissue treated three days in labelled proline. (Average weight per explant, 24 mg.). B. Tissue treated three days with C^{14}-proline and then grown eight more days in media free of C^{14}. (Average weight per explant, 72 mg.)

During the actual absorption period of 3 days 4 per cent. of the total C^{14} absorbed appeared each day as $C^{14}O_2$. After the tissue was removed to a medium free from C^{14}-proline the percentage of the previously absorbed radioactivity, which appeared in the carbon dioxide, declined so that the daily loss of C^{14} was less than 0·5 per cent. of the radioactivity in the tissue. This is shown in the Fig. 4C. The data in Figs. 3 and 4 clearly imply that once C^{14}-proline is incorporated into the protein of the tissue cultures it is not further metabolized except by progressive conversion to hydroxyproline. Any C^{14} which emerged as $C^{14}O_2$ could have come from either the C^{14}-labelled compounds in the soluble nitrogen of the tissue or from the very small amount of glutamic acid which was labelled in the protein (see Fig. 3).

All the evidence thus far points to the possibility that proline is oxidized to hydroxyproline *after* it is incorporated into the alcohol insoluble protein. However, the above experimental treatment with C^{14}-proline involved a relatively long period (i.e. 3 days), and at the end of this period there was almost as much C^{14} present in the tissue in the form of hydroxyproline as of

proline. It is reasonable, therefore, to assume that much of the oxidation of proline to hydroxyproline took place before the first analyses leading to Fig. 4A were made. A further experiment was carried out to demonstrate the incorporation of C^{14}-proline into the protein of the tissue during very much

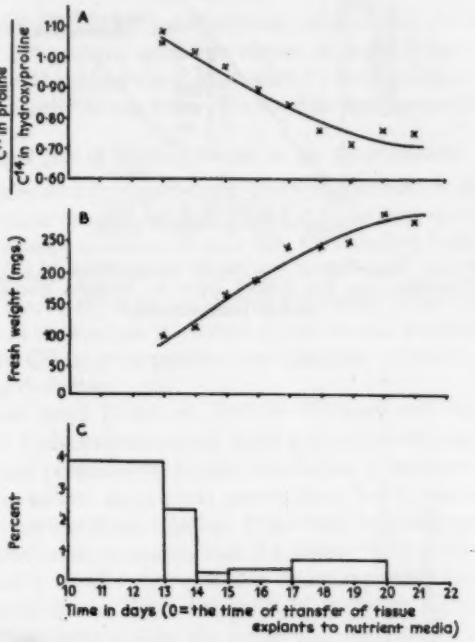

FIG. 4. Carrot explants were grown aseptically for 10 days in medium containing coconut milk. During a subsequent 3 days (day 10 to day 13) C^{14}-proline was also present in the medium. Subsequently (13 to 20 days) the growing tissue was in medium free from C^{14}-proline. A. Change in C^{14}-proline/C^{14}-hydroxyproline ratio with time. B. Change in fresh weight with time. C. Daily loss $C^{14}O_2$ as percentage of C^{14} originally absorbed by the tissue.

shorter periods and to determine the rate of conversion to hydroxyproline. For this purpose 50 carrot explants were again used and they were grown for 10 days in complete nutrient medium. At that point the explants were transferred to 10 ml. of nutrient media containing 25 μc. of C^{14}-proline in a Petri dish. During the absorption period the cultures were gently agitated. Subsequently tissue samples were taken for analysis after periods of exposure to the C^{14}-proline of 0·5, 1, 2, 4, and 24 hours respectively with the results shown in Fig. 5.

Fig. 5 shows that C^{14} from proline appeared in the protein in as little as 15 minutes, and thereafter the amount of radioactivity in the protein increased progressively with time. The ratio of C^{14} as proline to C^{14} as hydroxyproline

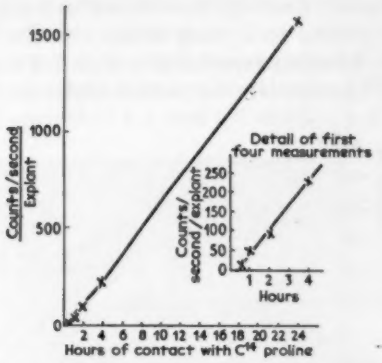

FIG. 5. The effect of time on the incorporation of C^{14} from C^{14}-proline into the alcohol insoluble nitrogen fraction of carrot-tissue cultures.

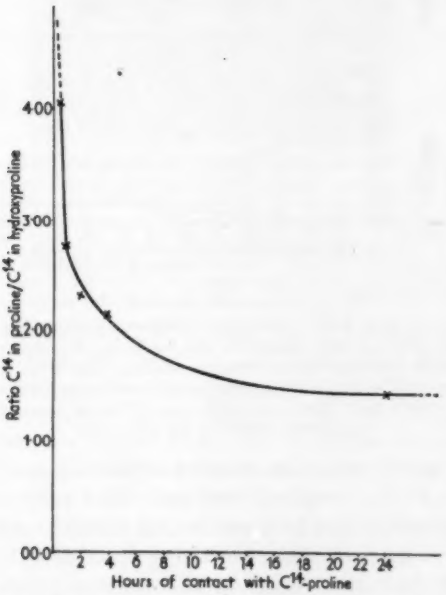

FIG. 6. The effect of time on the conversion of C^{14}-proline to C^{14}-hydroxyproline by carrot-tissue cultures.

in the protein was again determined by the same procedure. Fig. 6 shows the way in which this ratio changed with time, and that the rate of conversion of combined C^{14}-proline to C^{14}-hydroxyproline is rapid at the outset and falls

off with time. If, in Fig. 6, the ratio of C^{14} as proline to C^{14} as hydroxyproline is extrapolated toward zero time, it appears that this ratio approaches infinity, thus indicating that proline enters the protein molecule intact and is subsequently, though only partially and somewhat slowly, oxidized to hydroxyproline.

Whereas at the instant of incorporation virtually all the radioactivity is in the form of C^{14}-proline, after the elapse of some 8 days approximately one-third of the C^{14}-proline remains as such in the combined form, whereas approximately two-thirds has been converted to hydroxyproline.

The absorption and fate of hydroxyproline in the tissue cultures

All the evidence and interpretations given above rest on the presumption that hydroxyproline cannot be used directly in protein synthesis, although proline itself is readily so used. To test this, C^{14}-labelled hydroxyproline was necessary. This was isolated in small amounts from protein hydrolysates prepared from carrot explants which had previously been treated with C^{14}-labelled proline in the manner described above. In this way enough chromatographically pure C^{14}-hydroxyproline was obtained to carry out the experiment now to be described.

From previous work (Steward, Pollard, Witkop, and Patchett, 1958) it was known that hydroxyproline was itself markedly inhibitory to growth of carrot tissue, and presumably protein synthesis, at a concentration in the external solution as low as 10 parts per million; but it was also known that proline could overcome this inhibition. Therefore, sufficient proline was added to the external solution to ensure that the tissue could grow and synthesize protein sufficiently so that any possible hydroxyproline incorporation into the protein would not be limited by lack of synthesis. After three days of exposure to C^{14}-hydroxyproline the tissue was analysed in the manner that has been described. Table I demonstrates that hydroxyproline, like proline, could enter this growing tissue, for this amino-acid appeared on the chromatogram of the alcohol soluble fraction as the only spot both detectable with ninhydrin and also radioactive on the corresponding radioautograph. As expected, the protein hydrolysate revealed both proline and hydroxyproline, but, somewhat unexpectedly, *both* of these amino-acids as they occurred combined in the protein were *slightly radioactive*. Quantitative measurements of radioactivity showed that 97 per cent. of the radioactive hydroxyproline which had been absorbed by the tissue after 72 hours, remained in the alcohol-soluble nitrogen fraction: the 3 per cent. which was incorporated into the protein was distributed between proline and hydroxyproline in the ratio of 7:10.

The presumption is that some free hydroxyproline can be slowly reduced to proline in the tissue, and this is then incorporated as proline into the protein as rapidly as it is formed. The ratio (0·7) of C^{14} as proline to C^{14} as hydroxyproline in this protein is consistent with the view that the radioactivity enters the protein in the form of labelled proline rather than labelled

hydroxyproline (cf. Fig. 4A). For all practical purposes, therefore, one may conclude that hydroxyproline is *not* incorporated into protein as such in this tissue, and this is consistent with its role as a competitive inhibitor for proline which has been described elsewhere (Steward, Pollard, Witkop, and Patchett, 1958).

The results and interpretation given above require a method of conversion of free hydroxyproline to free proline. This may either occur via some as yet undisclosed method of direct reduction or by a sequence via pyrrole-2-carboxylic acid which has been shown by Radhakrishnan and Meister (1957) to be formed enzymatically from hydroxyproline. In turn the pyrrole-2-carboxylic acid could be reduced to proline.

If this reduction occurred readily, pyrrole-2-carboxylic acid should antagonize the growth inhibitory effect of hydroxyproline; this seems not to be so on the basis of the tests that have been made in this laboratory following the work of Steward, Pollard, Witkop, and Patchett (1958).

TABLE I

Distribution of C^{14} from C^{14}-hydroxyproline supplied to carrot-tissue explants

(*All data in terms of counts/sec. culture tube containing 5 carrot explants*)

	counts/sec.
Initial supply of hydroxyproline in media (0·5 μc.)	1,870
Residual hydroxyproline still in media after 72 hours	530
Hydroxyproline absorbed	1,340
Amount of C^{14} in $C^{14}O_2$ respired	0·0
Total amount of C^{14} in alcohol-soluble fraction*	1,102
Total amount of C^{14} in alcohol-insoluble fraction†	42
Amount of C^{14} unaccounted for (humin, &c.)	196

* By chromatography and radioautography *all* this radioactivity existed in hydroxyproline, the only radioactive compound detected.

† By chromatography and radioautography of the hydrolysate of this fraction *all* this radioactivity was present as proline and hydroxyproline in the proportions expected from Fig. 4A, where C^{14}-proline was the source of these amino-acids.

DISCUSSION OF THE RESULTS

The results of this investigation need to be considered against the general background of knowledge of the biochemistry of proline and of hydroxyproline, and also with special reference to our knowledge of protein synthesis and metabolism in the growing cells of carrot-tissue cultures.

Whereas all proteins contain proline, hydroxyproline is most conspicuous in collagen, and it has recently been noted as a conspicuous protein constituent in a variety of situations in which plant cells have been induced to proliferate in a random fashion and at a rapid rate. The presence of hydroxyproline in the bulk protein of proliferating cells gave rise to the idea that such cells may contain a distinctive kind or kinds of protein. These facts first aroused an interest in this protein constituent of carrot tissue, recognizing its similarity to collagen with respect to its hydroxyproline content. In the case of collagen

the conversion to hydroxyproline is thought to occur after the proline has been incorporated into the protein (Stetten and Schoenheimer, 1944; Stetten, 1949). Recently Smith and Jackson (1956), using methods very similar to ours though on animal tissue cultures, showed conversion of C^{14}-proline to C^{14}-hydroxyproline in collagen. However, the conversion was much slower than in carrot explants, reaching about 1 per cent. in 24 hours and 5 per cent. in 5 days. The subsequent conversion of proline to hydroxyproline after its incorporation into protein also explains the earlier observations made on carrot tissues in this laboratory and also the data in this paper.

Both proline and hydroxyproline are rapidly absorbed under aseptic conditions by actively growing carrot-tissue explants. However, 97 per cent. of the hydroxyproline remains as such in the alcohol-extractable portion. The 3 per cent. of hydroxyproline which was incorporated into protein in 3 days probably was first reduced to proline, thus requiring a means of conversion of free hydroxyproline in the tissue to proline.

The rate of incorporation of carbon from proline is unexpectedly rapid when compared with that from glutamine, γ-aminobutyric acid, or even glucose. Radioactive carbon supplied as proline can be detected in the protein 15 minutes after its addition to the external solution. Since the ratio of C^{14} in proline to C^{14} in hydroxyproline is initially high (4·0 after 30 minutes) and falls over a period of days to a stable value of about 0·70, it appears that proline first enters the protein as such, thereafter being progressively, though only partially, oxidized to hydroxyproline.

Recognizing that proline is generally convertible to glutamic acid via glutamic semialdehyde (cf. Vogel and Bonner, 1954) it is not surprising that some of the carbon from proline found its way into other soluble carbon compounds, although these compounds did not accumulate. While low levels of radioactive glutamic acid and glutamine were detected in the soluble fraction, it seems likely that carbon from proline never accumulates in these molecules but rather is quickly degraded to carbon dioxide and is never incorporated into protein. In other words, the proline which enters the cell is either incorporated *immediately* into protein or, escaping this fate, it is respired away. The fact that the bulk of the proline appears as protein rather than as carbon dioxide suggests that protein synthesis, or at least the synthesis of the proline-containing protein, occurs in the non-particulate cytoplasmic fraction before the proline ever gets to the principal site of the respiratory process, namely the mitochondrial particles.

While the evidence presented here clearly proves that free proline of the cell enters protein directly and is converted thereafter to hydroxyproline, the significance of the final ratio of proline to derived hydroxyproline is not known. This may have some meaning in terms of the structure of the protein in which this process occurs, but until such a protein has been purified and studied structurally, further speculation is hardly profitable. Nevertheless, the analogy has been drawn between the composition of collagen, the hydroxyproline-rich, metabolically inert, protein in the animal body, and this

hydroxyproline-containing plant protein fraction which, though present in rapidly growing cells, is also metabolically quite inert.

The possible presence in the non-particulate portion of the protoplasm of these cells of specific proteins with distinctive structural or physical properties is suggested by the appearance of the discrete cytoplasmic strands which occur in these cells and by the active protoplasmic streaming through and along them. Also, the rapidity of cell-division under the influence of growth factors in coconut milk might well be associated with distinctive kinds of protein which are active during the division. There is, at this time, no basis for assigning any specific role to the hydroxyproline containing, and metabolically inert protein of growing carrot cultures, except the fact of its characteristic presence in such dividing cells. Indeed, the protein which is synthesized in cells at the surface of cut disks of carrot root or potato tuber, in moist air, has also been found to be enriched with hydroxyproline. Thus cells which would eventually divide under the normal wound healing stimulus are similar in this respect to cells which divide under the influence of the coconut milk. However, in the slime mould *Physarum* protoplasmic streaming and osmotic work have been associated with an A.T.P. sensitive protein called myxomyosin, which has been isolated (Ts'o *et al.*, 1956).

Myxomyosin is known to be of high molecular weight (order of 6 million), to be associated with RNA, and is believed to split phosphate from A.T.P. As yet, nothing comparable is known of the physical properties of the inert, hydroxyproline-containing protein here described, but its inert nature and very presence in cells in which such active streaming and division may be observed suggested that it may be distinctively concerned with cytoplasmic structure rather than with metabolism.

Experiments have also been made in which the tissue has been allowed to metabolize for long periods, subsequent to the incorporation of C¹⁴-proline into the protein. The evidence presented is very striking testimony to the fact that, *once incorporated into protein* in this way, the proline undergoes no further change *except by conversion to hydroxyproline*, nor does it contribute appreciably to the carbon dioxide which is respired. In this respect the carbon which enters the protein from proline stands in sharp contrast to the carbon which appears in certain other protein amino-acids, particularly glutamic acid, the primary source of which was glucose.

These results, therefore, are compatible with the earlier interpretation (Steward, Bidwell, and Yemm, 1956) of the metabolism, respiration and protein synthesis in the growing cells of the carrot-tissue cultures. This explanation postulated two types, or methods, of protein synthesis which were distinguished by the source from which the carbon was drawn and the susceptibility of the protein so synthesized to 'turn over'. The results here reported, which establish the formation of a protein which incorporates proline directly and which does not metabolically 'turn over', confirm that part of the earlier hypothesis which related to the formation of a non-metabolized protein fraction which incorporated proline directly.

While the principal work here reported has been done with carrot tissue cultures, cultured potato tissue shows a similar behaviour, at least with respect to the incorporation of C^{14}-proline into the protein and its subsequent conversion to hydroxyproline.

Further progress along this interesting line will require the more exact characterization of the hydroxyproline-containing protein fraction. Work towards this end is in fact in progress. Preparatory to this further work, however, the principal location of this protein in the cell has been tested; and it was found that in large part it occurred in the non-particulate matter which remained after cell homogenates were centrifuged at 100,000 *g*. for 2 hours, as in the isolation of microsomes and mitochondria. Thus, in the consideration of the metabolism of the cell in terms of phases or compartments, it is noteworthy that this rapidly synthesized and characteristic kind of protein which contains hydroxyproline derived from proline is present in the soluble, non-particulate part of the cytoplasm. The extent to which the protein which 'turns over' in which hydroxyproline is not conspicuous may be a feature of the particulate inclusions in the cell involves other experimental work which is to be published later.

ACKNOWLEDGEMENTS

This investigation is part of a programme of research which is supported by a grant, C1357 (C6) and (C6S), to one of us (F. C. S.), from the National Cancer Institute of the Department of Health, Education, and Welfare, National Institutes of Health. This made possible the special assistance with the aseptic culture methods by Miss K. Mears, and with the analytical procedures by Mrs. R. Greenwood. This is gratefully acknowledged.

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The Uptake of Growth Substances

I. FACTORS CONTROLLING THE UPTAKE OF PHENOXYACETIC ACIDS BY *LEMNA MINOR*

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Received 10 July 1958

SUMMARY

An analysis has been made of the factors controlling the uptake of 2:4-dichlorophenoxyacetic acid (2:4-D) and related compounds by *Lemna minor*, grown under controlled conditions. All compounds were labelled with ^{14}C in the carboxyl group and the ^{14}C in the plants, after liquid combustion, assayed as barium carbonate.

With 2:4-D the rate of entry from concentrations of 8 to 32 mg./l. is maximal in the first 20 minutes, then falls progressively until the rate is zero between 1 and 2 hours and in the third phase (up to 24 hours) there is a net loss from the plants due to an outward movement of the compound back into the external solution. When the chlorine substitutions are in the 2:6-position the course of uptake is similar, save that loss to the solution takes place later. For the 2-chloro-substitution, after the initial phase of uptake there is some loss but this is followed by a period of recovery and uptake again proceeds, but slowly. In contrast, the course of uptake over 24 hours of phenoxyacetic acid is normal, i.e. there is a steady accumulation.

When plants are pretreated with labelled 2:4-D for 30–60 minutes and transferred to water or culture solution then over 90 per cent. of the ^{14}C is found in the external medium after 4·5 hours and this release cannot be accounted for in terms of exchange processes since the addition of unlabelled compound to the medium *retards* the rate of loss. This loss is slowed but not stopped at 1·25° C. and up to 22·5° C. the range of the Q_{10} is 1·6–1·9.

Uptake in the first 30 minutes is temperature sensitive between 7·5 and 30° C. (Q_{10} 2·3–2·6) and in general is positively and curvilinearly related to the external concentration. Pretreatment with *unlabelled* compound up to 2 hours progressively depresses the subsequent initial uptake of *labelled* growth regulator. It is concluded that initially the rate of entry greatly exceeds the rate of loss but that with time the ratio steadily diminishes to less than unity.

Initial uptake of 2:4-D is markedly dependent on the pH of the solution and closely but not completely correlated with the external concentration of undissociated molecules. On the other hand, the outward movement is relatively unaffected by the external pH.

Combinations of concentration and time of exposure which bring about loss to the solution need not cause any permanent retardation of growth. In fact, exposure for 1 hour to 8 mg./l. significantly accelerated growth in the next 8 days.

These results are discussed in relation to previous findings and it is concluded

that the pattern of uptake is determined on the one hand by the chemical structure of the growth substance and on the other by specific physiological differences at cell level.

INTRODUCTION

ALTHOUGH many aspects of the physiology of growth substances have been intensively investigated in the last decade, the factors controlling uptake have received surprisingly little attention. When Reinhold in 1954 published the results of the earliest investigations in this laboratory on the uptake of indole-3-acetic acid by segments of pea epicotyl and carrot disks she was able to cite only three previous papers which were directly concerned with the absorption of this auxin. More recently Andreae and van Ysselstein (1956) and van de Westeringh (1957) have further investigated the uptake of indole compounds by pea segments.

At the start of the present series of experiments in 1954 on the uptake of 2:4-dichlorophenoxyacetic acid and related compounds there was a complete lack of published information. Subsequently Johnson and Bonner (1956) described experiments on the absorption of 2:4-D by coleoptile sections of *Avena sativa* while Wedding and Erickson (1957) have investigated the influence of the external hydrogen ion concentration on the uptake of the same growth regulator by *Chlorella*.

The main aim of this investigation has been to elucidate the factors controlling uptake by intact higher plants. This first paper is concerned with the absorption of phenoxyacetic acids by the aquatic plant *Lemna minor*, this will be followed by an account of the uptake of 2:3:5-triiodobenzoic acid by the same species while current research relates to the absorption through the roots of 2:4-D by a wide range of species. Some of the results have been briefly reported (Blackman, 1956, 1958).

EXPERIMENTAL METHODS

Experimental procedure. The same clonal strain and the general methods of culture described by Blackman and Robertson-Cunninghame (1955) have been employed. Briefly the stock plants were grown in a solution containing trace elements developed by Steinberg (1941) but in order to increase the buffering capacity the phosphate content was doubled or quadrupled. The pH of the solution was adjusted to 5·1 which was optimal for growth. The plants were transferred to fresh solution every second day and thoroughly washed to reduce algal contamination to a minimum. The plants in either glass or plastic (polystyrene) containers were placed in a water bath held at 24° or 25° C. and illuminated continuously with 'Osram' daylight fluorescent tubes. In the earlier experiments the light intensity was maintained at 275 foot candles but subsequently raised to 300. Under these conditions the gain in dry weight is 21–23 per cent. per day.

In order to attain the necessary sensitivity of estimation compounds labelled in the carboxyl group with ^{14}C were synthesized and on the grounds

of economy there was therefore a need to use small samples of plants and small volumes of solution. For the purpose of accuracy of assay it was found that a minimum of 20 mg. of dried material was required and that in short-term experiments this requirement could be met if each replicate consisted initially of 115–20 fronds. To minimize variations between these samples the night before the experiment started the stock population was sorted into classes containing 2, 3, 4, and 5 fronds per plant and the next morning the same number of plants in each class were allotted to each treatment. The proportion of plants varied slightly between experiments but the ratio was of the order 10:10:15:1. By this procedure the coefficient of variation in the weight of the initial samples could be kept down to 2–3 per cent.

After investigation it was concluded that for experiments lasting up to a few hours the minimal satisfactory volume for the standard number of fronds was 23–25 ml., provided that the concentration of the growth regulator in the culture solution exceeded 8 mg./l.: for lower concentrations the volume was doubled. Tests showed that when the beakers containing the fronds and solution were returned to the bath (24–25° C. and 275–300 foot-candles) the loss of water by evaporation and transpiration did not amount to more than 5 per cent. at the end of 4 hours. If the beakers were covered with a thin glass sheet the loss at 30 hours was 21 per cent. Therefore if the experimental period exceeded 4 hours the beakers were either covered with glass sheets or more generally the volume was quadrupled to 100 ml.

To assess the changes with time in the external concentration of the growth regulator besides determining the amount taken up by the fronds the content of the culture solution was also analysed. When the experimental period was short the depletion of the solution due to uptake was never more than 5 per cent. even when the volume was minimal. Over longer periods, e.g. 2–4 hours, the external concentration remained relatively unchanged since the depletion due to uptake was more than offset by the slight degree of concentration resulting from evaporation.

Previous findings (Blackman and Robertson-Cunninghame, 1953) had demonstrated the importance of maintaining the pH of the external solution constant and the drift of pH with time was examined. Up to 4 hours the change was 0·1 units per hour. If the experimental period was longer, a duplicate experiment was set up using unlabelled growth regulator, and at the appropriate intervals the quantities of dilute acid required to bring the solutions back to the original pH determined. These quantities of acid were then added to the corresponding solutions containing the radioactive material.

Before the experimental samples were dried, weighed, and analysed it was essential to ensure that all the radioactive solution was washed off the plants. To discover how many washings in water were required two procedures were adopted. In one case the fronds were dipped into a solution containing 16 mg./l. ^{14}C labelled 2:4-D for approximately 30 seconds and then washed for a few seconds in a varying number of changes of tap water. In the second series the fronds were left for 1 hour in two concentrations (16 and 60 mg./l.)

before washing. After washing surplus water was removed with blotting-paper and the fronds transferred to small glass tubes, which were then placed in an oven at 96–98° C. for 12 hours. Subsequent analysis showed that in both series plants washed up to four times contained the same amount of labelled carbon as plants washed once. It was therefore decided to wash all samples at least twice and the procedure was standardized so that between taking the sample from the original solution and placing it in the oven the period was 10 minutes.

In the initial experiments the effects of the treatments on the fresh weight were also ascertained. After washing, but before drying, the sample was transferred to a pad of cotton-wool which was then placed in a centrifuge tube and spun at 3,400 r.p.m. for 5 minutes. The plants, which could be readily picked off the cotton-wool, were then weighed immediately.

Method of ^{14}*Carbon assay.* Using a liquid combustion procedure the carbon in the dried plant material was converted to barium carbonate and the ^{14}C assayed, employing an end-window Gieger counter.

The oxidizing mixture was that developed by van Slyke *et al.* (1951) and the apparatus was a modification of the one designed by Claycomb and his collaborators (1950). The products of the oxidation were condensed in a trap at −180° C., the carbon dioxide was then evaporated by warming to −70° C. and absorbed in a standard barium hydroxide solution. These operations were carried out under reduced pressure. The suspension of barium carbonate was neutralized with standard hydrochloric acid and phenolphthalein as the indicator and filtered onto a disk of filter paper which could be secured in a planchet by means of a small brass ring. The carbon content of the sample was obtained from the titration data. The counter geometry was such that a sample of specific activity 1 mμc./mM. carbon gave a count of 7·4 cts./min. above background. All samples were of infinite thickness or corrected thereto.

Chromatography conditions. All chromatograms were run on Whatman No. 4 paper using a mixture of *iso*propanol (10 parts), water (1 part), and ammonia d. 0·88 (1 part) as the developing solvent. The radioactivity was recorded by means of an automatic device constructed in this Department (Loughman and Martin, 1957).

Preparation of ^{14}*C labelled phenoxyacetic acids.* These were all prepared by the same general procedure. Bromoacetic acid-1-C14, obtainable commercially, was reacted with an excess of the required phenol in the presence of the correct amount of sodium hydroxide solution. To facilitate the manipulation of small quantities the following procedure was adopted:

Sodium hydroxide (1·4 mM.) as a water solution was evaporated to dryness in a short length of approximately 0·6-cm.-bore glass-tubing by attachment of the latter to a good vacuum system. The phenol (1·2 mM.) was added as a solution in ether and the ether removed by evaporation under vacuum. The bromoacetic acid (0·2 mM.) in water (0·3 ml.) was next added and the tube sealed. If dilution of the bromoacetic acid-1-C14 with inactive material

was necessary then the active material was washed into the reaction tube with a solution of inactive bromacetic acid (0·2 ml.) of the requisite concentration and 0·1 ml. of water. The mixture was heated in a water bath at 100° C. for 6 hours.

The contents of the tube were then washed into a boiling-tube using water (3 ml.) and saturated with carbon dioxide. The excess of phenol was extracted with ether and the aqueous residue made acid with hydrochloric acid. Further extraction with ether followed by evaporation yielded the phenoxyacetic acid in almost quantitative yield. Crystalization was effected from a mixture of benzene and light petroleum (bp. 40–60° C.).

In this way were prepared:

2:4-dichlorophenoxyacetic acid sp. act. 5 mc./mM. mp. 138° C.
2:6-dichlorophenoxyacetic acid sp. act. 5 mc./mM. mp. 132° C.
2-chlorophenoxyacetic acid sp. act. 5 mc./mM. mp. 144° C.
phenoxyacetic acid sp. act. 5 mc./mM. mp. 98° C.

2:4-dichlorophenol was obtained especially pure from the Coalite and Chemical Company. 2:6-dichlorophenol was prepared by a previously described procedure (Tarbell, Wilson, and Fanta, 1955). 2-chlorophenol was obtained commercially and fractionally distilled. Phenol was the Analar grade.

EXPERIMENTAL RESULTS

The course of uptake. The influence of the external concentration and the length of treatment on the amount of growth regulator absorbed was examined. Selected batches of fronds were transferred to culture solutions containing the labelled compounds and returned to the same conditions of light and temperature to which they had previously been subjected. All experiments were carried out at 24–25° C.; in Expts. 1, 4, 5, and 6 the light intensity was 300 foot candles while in Expts. 2 and 3 the intensity was 275. In some experiments (2, 3, 4) the pH of 5·1 was the same as that of the stock cultures but in others (1, 5, 6) it was adjusted to 4·6.

The results of two experiments, selected from those designed to investigate the initial phase of the uptake of 2:4-D are shown in Fig. 1. It is seen that uptake is rapid in the first 10–20 minutes followed subsequently by a slight fall. Previous experiments on *L. minor* by Blackman and Robertson-Cunninghame (1953) had indicated that molecules of this growth regulator were preferentially absorbed over ions. Thus, since the pK is 2·9[1] it would be expected that the amounts absorbed in Expt. 1 at the lower pH would be greater.

Assuming that the difference in the fresh and dry weights is approximately equivalent to the total volume of the cells, excluding intercellular spaces, then the time taken for the mean internal concentration to equal the external concentration can be predicted and in this and subsequent figures an arrow

[1] This figure, determined by S. Novoa in this laboratory, is somewhat lower than the values usually quoted by other workers.

indicates the point when equilibrium is reached. In Expt. 1 irrespective of the external concentration equilibrium is attained after about 10 minutes. In Expt. 2, where with the higher pH the rate of uptake is slower, there is an inverse relationship with concentration. At the two lowest concentrations equilibrium is not attained at the end of 60 minutes but at the highest concentration of 64 mg./l. equilibrium is reached after 17 minutes.

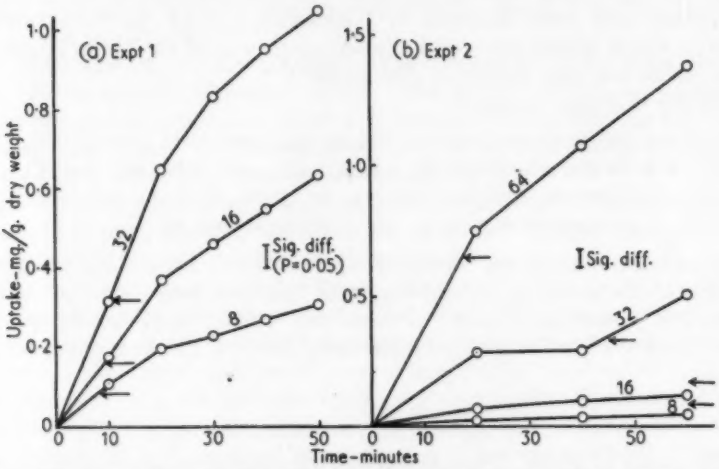

FIG. 1. The course of uptake of 2:4-D from different concentrations (indicated on curves in mg./l.) within the first hour. The pH of the external concentration was 4·6 in Exp. 1 and 5·1 in Expt. 2. In this and subsequent figures the arrows indicate when the calculated mean internal concentration of the whole plant equals the external.

While the course of uptake within the first hour follows the general pattern of the absorption of inorganic ions, for longer periods the results are to a marked degree anomalous. From Fig. 2*a* it is apparent that uptake is rapid in the first hour, slows down in the second, and in the next 2 hours there is a net loss of labelled carbon from the plants treated with 16–32 mg./l. If the period of treatment is extended to 24 hours (Fig. 2*b*) then after 4 hours there is no further apparent uptake over the concentration range of 4 to 16 mg./l., while at 32 mg./l. there is a progressive loss. It should also be noted that only in Expt. 4 and then only for the highest concentration does the mean internal concentration exceed the external.

In Figs. 1 and 2 uptake has been expressed in terms of the dry weight and it seemed pertinent to determine whether the treatments had any differential effects on the absorption of water and therefore an alteration in water content. In another experiment of similar design to Expt. 4 the fresh weights at 4, 12, and 24 hours were recorded. Employing the method already described on p. 36 no significant differences in water content (mean 92 per cent.) could be detected for plants treated with 4 to 44 mg./l. Since the time of handling

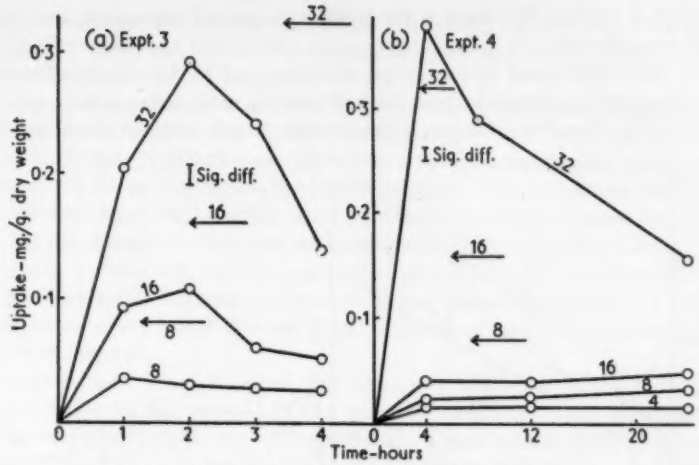

FIG. 2. The course of uptake of 2:4-D from different concentrations (indicated on curves in mg./l.) within (*a*) the first 4 hours, (*b*) 24 hours.

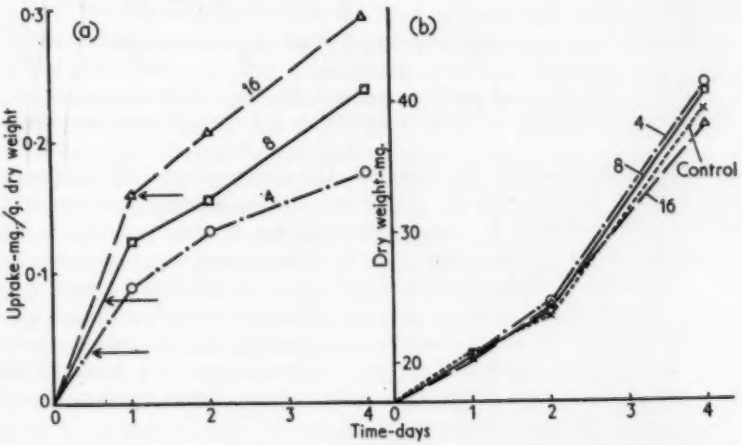

FIG. 3. Expt. 6. The course of (*a*) uptake, and (*b*) growth over 4 days with different concentrations (shown as mg./l.) of 2:4-D.

exceeded 10 minutes it is probable that the technique was not sensitive enough to show up small differences but the maximum difference recorded between extremes was only 1 to 2 per cent.

The next step in examining the course of uptake was to extend the experimental period even farther and the pattern over 4 days is shown in Fig. 3. After the first day the increase in content falls off at each concentration: that is, the net gain does not keep pace with the production of dry matter. From

Fig. 3*b* it is evident that none of the treatments caused any significant change in the growth-rate.

The last experiment in this series was designed to discover how far this anomalous course of uptake was also induced by other substituted phenoxyacetic acids. For this purpose, phenoxyacetic acid, and the 2-chloro- and

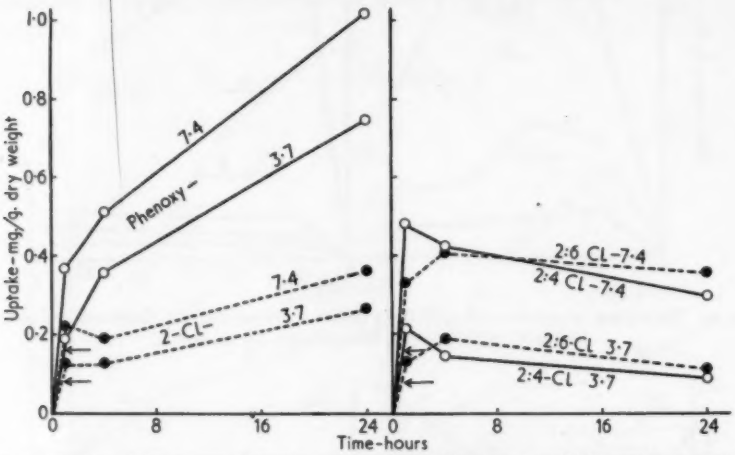

FIG. 4. Expt. 7. The course of uptake over 24 hours from $3{\cdot}7 \times 10^{-5}$ M. and $7{\cdot}4 \times 10^{-5}$ M. phenoxyacetic acid and the 2-chloro-, 2:4-dichloro-, and 2:6-dichloro-substitutions.

2:6-dichloro-substitutions were selected. The results given in Fig. 4 demonstrate that the trend is markedly dependent on the chemical structure. With phenoxyacetic acid there is a steady accumulation during the 24 hours. The introduction of one chlorine atom leads to a slower rate of uptake in the first hour, in the second uptake is markedly checked but recovers between 4 and 24 hours. Double substitution by chlorine in the 2:6- as against the 2:4-position leads to the same salient characteristics, namely that the initial phase of uptake is followed by a period of net loss. There is, however, a distinct difference between these two compounds since initially active uptake proceeds for longer with the 2:6-chloro-compound.

Factors determining the course of uptake. In seeking to analyse how an initial positive uptake could change with time to an apparent negative rate it seemed that there were two possible explanations. Weintraub, Brown, Fields, and Rohan (1952) have shown that when bean plants are treated with labelled 2:4-D, $^{14}CO_2$ is subsequently slowly released. It could therefore be advanced that in *L. minor* after an initial rapid uptake the rate slows down until the amount of labelled carbon entering the plant is less than the amount lost as $^{14}CO_2$. To account for the results of Expt. 6 it must also be postulated that phenoxyacetic acid itself is not metabolized in this way and that the rate or type of decomposition of the remaining compounds varies with the structure.

Alternatively, it could be postulated that the growth regulator or a metabolic product is transferred back to the external solution and that the rate of release varies with time.

On general grounds it was concluded that this second mechanism was the more probable and it was decided to investigate this first. As an initial approach (Expt. 7) plants were placed in a solution containing 56 mg./l. of labelled 2:4-D for 0·75 hours, then some samples were withdrawn for immediate carbon assay while others were transferred to distilled water for 22, 44, 88, and 176 minutes before they were analysed. The results showed that after transference there was a progressive fall in the activity of the plants and that the distilled water became radioactive. If the initial content at the time of transference is expressed as a 100 then the loss was 34, 56, 77, and 90 for the four time intervals.

To determine to what extent the large loss after 176 minutes could be accounted for by the amount in the external medium, 10 ml. aliquots were taken, evaporated, and the ^{14}C estimated. These assays showed that the whole of the loss from the plants (24·99 μg.) was matched by an equivalent gain in the water.

This balance sheet was based on the assumption that it was the regulator itself and not some product that had been released into the water. To test whether this assumption was warranted approximately 80 ml. of the external medium was taken and after a few drops of N/100 KOH had been added evaporated down on a water bath to 15 μl. This residue was spotted on to chromatogram paper and the paper developed with *iso*propanol-ammonia-water (see p. 36). Using the automatic scanner only a single peak of radioactivity was recorded on the paper, while the Rf corresponded closely to that obtained from other simultaneous runs with the original solution of labelled growth regulator. In this connexion Andreae and Good (1957) have found that at the end of 24 hours, the 2:4-D absorbed by segments of pea epicotyl, except for a very small fraction, remains unchanged.

It was decided that the next logical step would be to determine whether entry or egress occurred simultaneously and whether the rate of release was dependent on the external concentration. The experimental procedure adopted was as follows: (1) batches of plants were placed in solutions containing 8, 16, and 32 mg./l. of *isotopically labelled* growth regulator (pH 4·6) and withdrawn for assay after 30, 90, and 270 minutes, (ii) batches were treated with 8 and 16 mg./l. of *labelled* material for 30 minutes and then transferred either to culture solution or culture solution containing double the initial concentration of *unlabelled* growth regulator, and samples again withdrawn at 30, 90, and 270 minutes, (iii) samples were placed for 30 minutes in 8 or 16 mg./l. of *unlabelled* compound and then transferred to 16 or 32 mg./l. of *labelled* compound and the same time sequence of sampling adopted.

Fig. 5 (Expt. 8) shows (*a*) the course of uptake without any pre-treatment, (*b*) the net uptake of the labelled compound after pre-treatment with the unlabelled compound, and (*c*) the loss of labelled compound after pretreatment

with the unlabelled compound. The results demonstrate unequivocally that at least after 30 minutes entry and egress are taking place simultaneously. There are two other features of interest. Firstly, there is a change with time from a positive to a negative rate of uptake when the external concentration is low but not when it is high (Fig. 5*a*). Secondly, that the rate of outward movement is diminished by the presence of unlabelled material in the external solution (Fig. 5*c*).

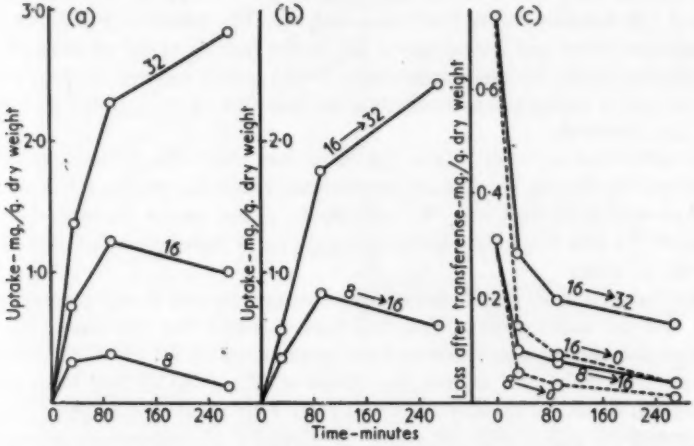

FIG. 5. Expt. 8. The course of uptake from 8, 16, and 32 mg./l. solutions of ^{14}C labelled 2:4-D (*a*) without any pretreatment, and (*b*) following pretreatment for 30 minutes with 8 and 16 mg./l. of *unlabelled* compound, and (*c*) the loss to the external solution, in the presence and absence of *unlabelled* growth regulator, of plants previously treated for 30 minutes with 8 and 16 mg./l. of *labelled* compound.

This effect of the external concentration on the outward movement was confirmed in other experiments and the results of one of these (Expt. 9) are given in Fig. 6. In this instance the plants were treated for 30 minutes with culture solutions (pH 4·6) containing either 16 or 32 mg./l. of labelled compound before they were transferred to culture solutions of the same pH, to which had been added 0, 16, and 32 mg./l. of unlabelled growth regulator. It is once more evident that the rate of loss is initially very rapid and that the higher the external concentration of the growth regulator the slower the outward movement, especially in the first half hour.

From this series of experiments it was concluded the course of uptake is dependent on the changes with time in the relative rates of entry and egress. In the initial phase the rate of entry will at first be controlled dynamically by physical factors of diffusion, adsorption, and exchange but that with time other factors come into play. In consequence, the relative rates of entry and egress are so changed that the net gain to the tissues is increasingly depressed until eventually there is a net loss.

On these assumptions it would be expected that if plants were pretreated with unlabelled growth regulator before they were transferred to a solution containing radioactive material then the subsequent rate of uptake would be governed by the duration of the pretreatment and that up to about 2 hours

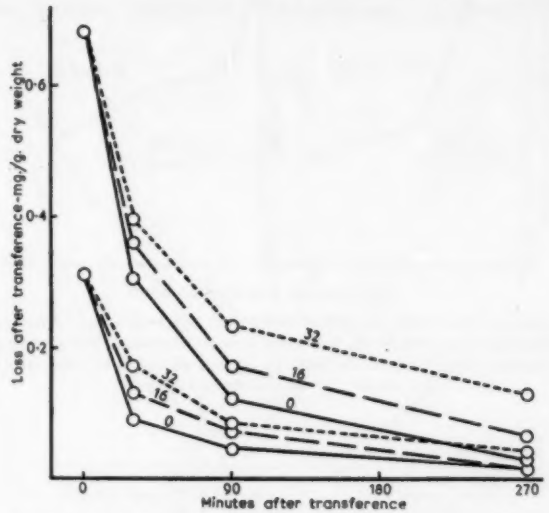

FIG. 6. Expt. 9. The effects of the external concentration of unlabelled 2:4-D on the rate of release of labelled compound from plants, pretreated for 30 minutes with concentrations of 16 and 32 mg./l.

uptake would fall progressively. That this assumption is correct is shown by the results of Expts. 10 and 11 (Fig. 7).

Influence of temperature on rates of entry and egress. Since the experimental evidence strongly suggested that the two components governing the net amount of growth regulator in the plant operated during the whole of the course of uptake it was not possible to devise experiments in which the two components were completely separated and in examining both the effects of temperature and the pH of the external solution some compromise had to be made. It seemed that for uptake the errors would be minimal if measurements were restricted to the initial phase when the rate of entry greatly exceeds the reverse outward movement and in studying the influence of temperature the experimental period was limited to the first 30 minutes.

The results of the most comprehensive experiment involving four temperatures and three external concentrations (pH 4·6) are given in Fig. 8. That there is a large effect of temperature at each concentration is very evident. In fact when linear regressions of the logarithm of uptake against temperature were fitted it was found that the temperature quotient for each concentration was similar, namely 2·6, 2·5, and 2·3 for 8, 16, and 32 mg./l. respectively.

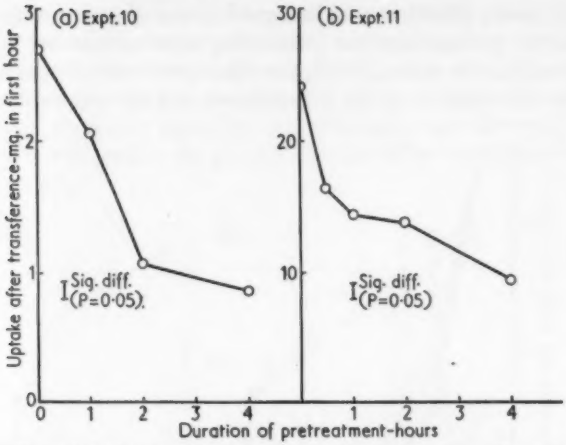

FIG. 7. The effect of the length of pretreatment with unlabelled 2:4-D on the subsequent uptake of the labelled compound in the first hour after transference. The concentration before and after transference was 16 mg./l. in Expt. 10 (*a*) and 48 mg./l. in Expt. 11 (*b*): the pH of the solutions was maintained at 5·1.

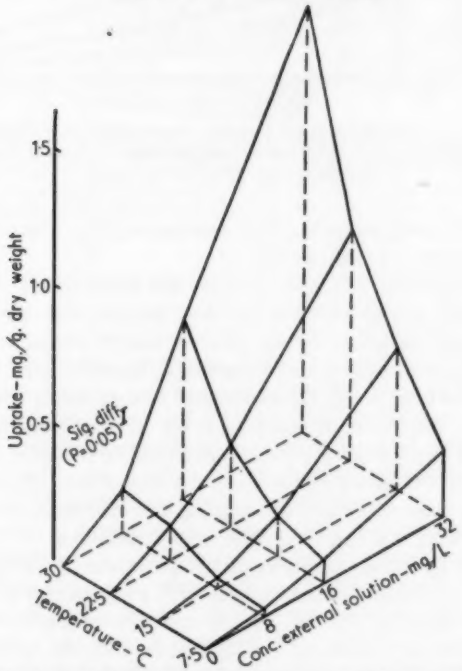

FIG. 8. Expt. 12. The interrelationship between temperature and concentration on the uptake of 2:4-D over 30 minutes.

There is another feature to note in Fig. 8: that is the relationship between uptake and the external concentration. At each temperature uptake increases in a curvilinear manner with concentration.

Similar experiments were undertaken to ascertain the influence of temperature on the outward movement. In experiment 13, plants maintained at

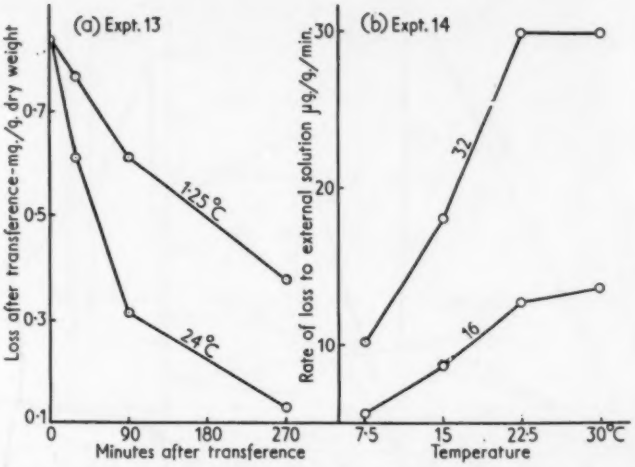

FIG. 9. The effects of temperature on loss to the external solutions of plants previously treated with labelled 2:4-D at (*a*) Expt. 13, 16 mg./l. for 2 hours, and (*b*) Expt. 14, 16, and 32 mg./l. for 30 minutes. In Expt. 14 the loss recorded was for the initial 30 minutes.

24° C., were allowed to take up labelled growth regulator for 2 hours from a concentration of 16 mg./l. and pH 5·1 before they were transferred to culture solution at either 24° C. or 1·25° C. and the loss from the tissues measured. From Fig. 9*a* it is evident that release to the solution is not inhibited at 1·25° C. Raising the temperature to 24° C. has a significant effect for the first 90 minutes (Q_{10} 1·6) but not afterwards.

In other experiments a wider range of temperatures was employed. For example, in Expt. 14 plants were first treated for 30 minutes at 25° C. with solutions of 16 and 32 mg./l. (pH 4·6) before they were transferred in batches to distilled water held at 7·5, 15, 22·5, and 30° C. and after 30 minutes removed for assay. It is evident from Fig. 9*b* that between 7·5 and 22·5° C. there is a marked response. For both levels of pretreatment the regression of the logarithm of the loss against temperature is linear and the temperature coefficients for the 16 and 32 mg./l. concentrations were 1·68 and 1·86 respectively. Above 22·5° C. temperature exerts a much smaller effect.

Influence of pH on the components of uptake. From an earlier investigation (Blackman and Robertson-Cuninghame, 1953) cogent but indirect evidence was forthcoming that the uptake of 2:4-D by *L. minor* was largely in the molecular form and therefore much influenced by the degree of dissociation

and the pH of the external medium. It therefore seemed pertinent to establish more precisely the relationships. As in the previous series of experiments the approach has been to separate as far as possible the effects of pH on the two components.

Expt. 16 was concerned with studying the initial phase of entry, and from

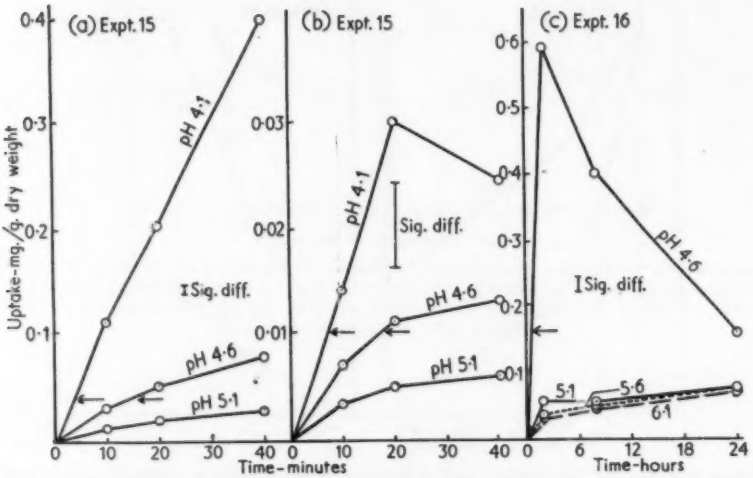

FIG. 10. The influence of the external hydrogen ion concentration on the uptake of 2:4-D (*a*) from 4 mg./l. solution during the first 40 minutes, (*b*) from 1 mg./l. solution during 40 minutes, and (*c*) from 16 mg./l. solution during 24 hours.

Fig. 10*a* and *b* it is seen that the amount taken up even in the first 10 minutes is strongly dependent on the external hydrogen ion concentration. Thus, in the most acid solutions the time taken to reach the point of equilibrium was only 7 and 4 minutes for the two concentrations of 1 and 7 mg./l.

It is also to be noted, more particularly in Fig. 10*c* (Expt. 17) that as the experimental period is extended there can be a dramatic change in the relationship between uptake and pH. For example, after the first 2 hours the amount absorbed at pH 4·6 as against 5·6 was 16 times as great: after 24 hours the ratio had declined to two.

Clearly in analysing this varying pattern the next step was to investigate the phase of outward movement. The procedure was to place batches of plants under constant conditions (300 foot-candles, 25° C., pH 4·6) and allow them to take up the labelled compound for 30 minutes before they were transferred to culture solutions of different pH and the loss from the plants measured at the end of 30 minutes.

Fig. 11 gives the results of Expt. 17 where there were three concentrations in the period of pretreatment. The effect of the pH of the external solution on the release of the growth regulator is very slight but there is nevertheless

a significant interaction. With an increase in pH the difference in trend for 32 and 16 mg./l. is significant.

The effects of concentration and time of exposure to 2:4-dichlorophenoxyacetic acid on the subsequent growth. In Expt. 5 (Fig. 3*b*) it was demonstrated that exposure to 4, 8, and 16 mg./l. of 2:4-D did not over a period of 4 days

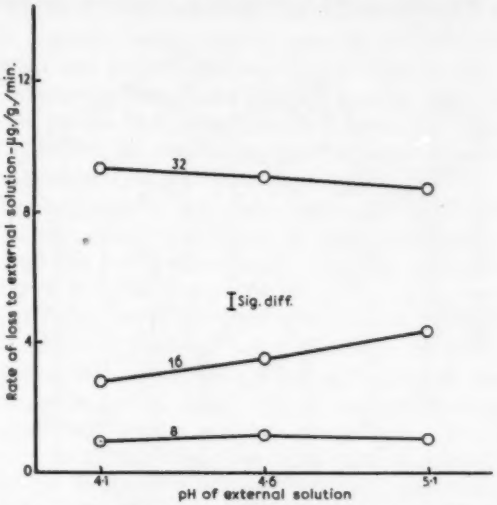

FIG. 11. Expt. 17. The influence of the external hydrogen ion concentration on the amount of labelled 2:4-D lost to the solution in 30 minutes, following a pretreatment with 8, 16, and 32 mg./l. (pH 4·6) of unlabelled compound for 30 minutes.

cause any significant change in the growth rate. From previous investigations (Blackman and Robertson-Cunninghame, 1955) it was known that the effect of this growth regulator is cumulative and that over longer periods these concentrations can cause a retardation of growth. None of the experiments were, however, concerned with the effects of short periods of exposure such as have been employed in many of the present experiments. This aspect was therefore investigated in a factorial experiment in which plants were treated for 1 to 8 hours with concentrations ranging from 4 to 112 mg./l. before they were transferred to normal culture solution and the dry weights determined after 8 days. During the whole of the experiment the pH of the solution was 5·1, the temperature 25° C. and the light intensity 300 foot-candles.

The results shown in Fig. 12 are in some ways unexpected. A combination of 8 mg./l. and an exposure time of 1 hour has lead to a significant *increase* in the growth-rate while even with 16 mg./l. and a 2-hour exposure there is clearly no inhibitory effect. In fact for a depression in the growth-rate to attain a significant level, a combination of either 32 mg./l. and 2 hours' exposure or 64 mg./l. for 1 hour is the minimal requirement.

DISCUSSION

Reinhold (1954) concluded that for carrot disks uptake of indole-3-acetic acid could be divided into two phases. Over the first 4 hours when uptake was relatively rapid the rate was controlled by physical factors while subsequently the much slower rate, which appeared to be independent of the external concentration, was determined by metabolic processes. Johnson and Bonner

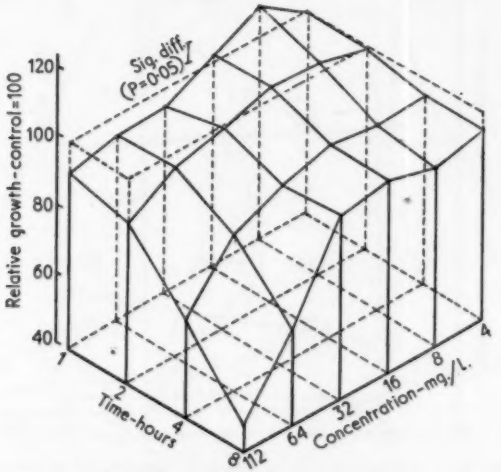

FIG. 12. Expt. 18. The effects of time of treatment and the concentration of 2:4-D on the subsequent growth over a period of 8 days.

(1956) also held that the uptake of 2:4-D by *Avena* coleoptile segments had physical and metabolic components, but that the physical processes operated for a much shorter time, of the order of half an hour, while in the subsequent metabolic phase the rate of uptake was limited by the external concentration. Similarly for *Chlorella*, Wedding and Erickson (1957) considered that for the first 20 minutes the rate was determined by a diffusion and adsorption equilibrium.

With etiolated segments of pea stem it would seem from the results of both van de Westeringh (1957) and Andreae and van Ysselstein (1956) that in the uptake of indoleacetic acid there is no clear-cut evidence for metabolic processes taking over from physical processes. It is true that the first measurements were not made until an hour after the segments had been placed in the solution but when van Westeringh's data are expressed in graphical form the rate of entry remains relatively constant over at least 4 hours and in the subsequent 20 hours only exhibits a slight but progressive fall.

In view of these diversities in the pattern of the course of uptake with time it is not surprising that the results for *L. minor* should again be different. In the first 20–30 minutes the trend resembles that for *Avena* segments and

Chlorella but subsequently there is no steady rate of accumulation but a progressive diminution until ultimately there is a net loss to the external solution.

The evidence of other workers that physical factors are more important in the initial period is of four kinds, namely the effects of enzyme inhibitors, the relationship between rate of uptake and external concentration, the influence of the external concentration on the loss to the external solution, and the magnitude of the temperature quotient. For *Avena* segments and carrot disks uptake is only partially suppressed by cyanide and iodoacetic acid while 2:4-dinitrophenol and *p*-chloromercuribenzoate depress the initial rate far less than the subsequent slower rate (Reinhold, 1954; Johnson and Bonner, 1956). When the period of treatment is 4·5–5 hours the uptake of auxin by pea segments is halved by cyanide (5×10^{-4} M.) and even more depressed by anaerobic conditions (van de Westeringh, 1957).

For both *Avena* coleoptiles and less conclusively for *Chlorella* the initial rate of entry has been shown to be almost directly proportional to the external concentration and this would be expected if diffusion phenomena dominated the mode of entry. For the later phase of uptake by *Avena* coleoptiles the curvilinear relationship was of the usual type that can be ascribed to physical adsorption and chemical binding. From Fig. 8 it has been seen that the amount entering in the first 30 minutes is linked positively but curvilinearly with the concentration and in view of this somewhat surprising trend it is of interest to examine the results of other experiments and these have been plotted in Fig. 13. In Expt. 2 (Fig. 13*a*) there is the same curvilinear relationship as in Expt. 12, in Expts. 8 and 19 (Fig. 13*b*) departures from linearity are slight while in Expt. 1 (Fig. 13*c*) the relationship is again curvilinear but of an opposite trend.

In comparing these results it should be recalled that while Expts. 2, 8, and 19 were carried out at pH 4·6 Expt. 2 was at pH 5·1. Therefore, in terms of the external concentration of undissociated molecules it can be calculated that at pH 5·1 as against 4·6 the effective concentration is reduced to a third and at these low concentrations the results are anomalous. In this connexion Scott Russell (1954) found that over a very wide concentration range of phosphate, absorption of this ion by barley roots followed a sigmoid pattern. Some of the results of Expt. 15, given in Table I, are also relevant. As the pH is increased from 4·1 to 5·1 and the percentage of undissociated molecules reduced from 5·8 to 0·6 so the relative uptake at the two concentrations is also altered. This change may well be associated with the shift in the degree of dissociation but it is also consistent with the assumption at that low concentrations small differences in concentration may result in disproportionate increases in the rate of uptake. What, however, is not consistent is the divergence in trend between experiment 1 and 12 (Figs. 13*c* and 8); the concentrations and pH were the same and for 30 minutes' exposure the differences in temperature could be as small as 2·5° C. (25 as against 22·5).

Turning to the nature of the outward movement to the external solution the results for *L. minor* are again at variance with the general pattern established

by previous workers. Both Johnson and Bonner (1956) and Wedding and Erickson (1957) showed that if, after an initial period of uptake, coleoptile segments or *Chlorella* were transferred to a solution containing no unlabelled growth regulator loss to the external solution reached equilibrium within 30 minutes and that the initial rate and the amount finally released were

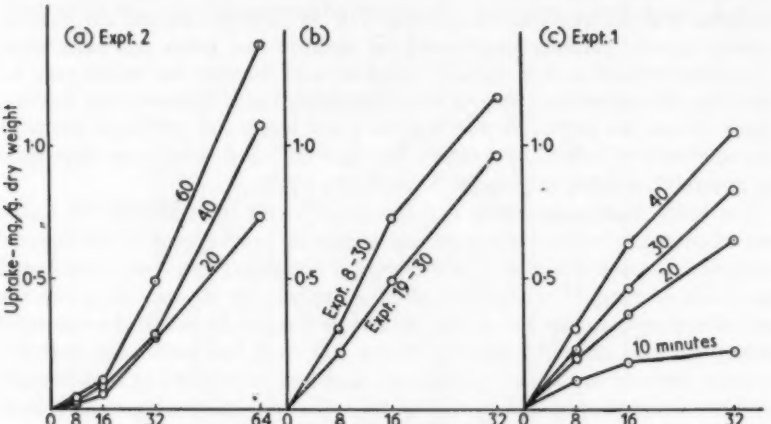

FIG. 13. The relationship between the concentration of 2:4-D and uptake over short periods shown on the curves in minutes.

TABLE I

The relationship between the rate of uptake of 2:4-D and the external concentration at different levels of hydrogen ion concentration

Time of treatment (minutes)	pH	Uptake (mg./g. dry weight) Concentration—mg./l.		Ratio of uptake at two concs.
		1	4	
10	4·1	0·014	0·111	7·9
	4·6	0·007	0·032	4·6
	5·1	0·004	0·014	3·5
20	4·1	0·030	0·207	6·9
	4·6	0·011	0·053	4·8
	5·1	0·005	0·017	3·4

increased when unlabelled 2:4-D was present in the external solution. These findings were compatible with the concepts of exchange between the tissues and the solution, but the present findings are aberrant. In the first place it has been demonstrated that following transference either to distilled water or culture solutions a very high proportion of the radioactivity is lost to the external solution within 240–70 minutes (Figs. 5*c*, 6, 9*a*). Secondly, that the rate of loss is not accelerated but is retarded by the presence of the unlabelled compound in the solution. At the present stage of the investigation it is diffi-

cult to provide a satisfactory explanation, especially in view of the fact that it will be shown in subsequent papers that the release of 2:3:5-triiodobenzoic acid by *L. minor* is not affected by the external concentration and that the loss of 2:4-D from segments of pea stem, although much greater than from *Avena* coleoptile segments, is enhanced by the presence of unlabelled growth regulator in the solution. It should also be noted that the movement of ^{14}C labelled alphamethoxyphenylacetic acid out of roots was not affected by including the unlabelled compound in the nutrient medium (Linder, Craig, and Walton, 1957).

It is reasonable to suppose that some exchange between the unlabelled and labelled growth regulator will be proceeding from the start of the transference of *L. minor* and therefore the specific activity of the ^{14}C in the tissues will be diluted but it seems unlikely that the degree of dilution could be great enough to account for the magnitude of the observed changes.

The evidence of Expt. 12 (Fig. 8) on the effects of temperature on uptake suggest that during the first 30 minutes metabolic processes play an important part in determining the rate and it would seem that the temperature coefficients are somewhat higher than those deduced from the data of Johnson and Bonner (1956) for *Avena* coleoptiles. On the other hand, the magnitudes of the temperature coefficients for Expts. 13 and 14 (Fig. 9) indicate that both physical and metabolic processes come into play. Indeed, from Fig. 9*a* the conclusion could be reached that the metabolic processes are more important during the initial period.

There is one other aspect of the factors controlling uptake which calls for comment and that is the influence of pH. From Fig. 10 and Table I it is evident that in the first 20 minutes uptake is much altered by the change in pH from 4·1 to 5·1, and the relationship between the rate of uptake and the shift in the degree of dissociation is brought out in Fig. 14. It is apparent that in the first 10 minutes uptake from the higher concentration is very closely linked with the external concentration of undissociated molecules but that at 1 mg./l. the relationship is less precise since the fall in the rate of uptake is small. At 20 minutes the differences between the two concentrations are less striking.

Since the external pH has a much smaller effect on the outward movement (Fig. 11) and since the relative rates of entry and egress change with time it is not unexpected that the relationship between net uptake and pH also varies with time (Fig. 10*c*).

Wedding and Erickson (1957) using a high concentration of 2:4-D (1×10^{-3} M.) established that between pH 4 and 7 the initial uptake of *Chlorella*, relative to the concentration of undissociated molecules, increases with rising pH and concluded that uptake is very largely in the form of the undissociated molecule. Thus, over the same range of pH, as in Expt. 16, the large changes in uptake were of the same order. The results obtained by Johnson and Bonner (1956) for *Avena* coleoptiles were quite different: even a change from pH 7·5 to 3·5 only caused the initial rate of uptake to increase

by a factor of 2·5. Similarly, Reinhold (1954) and van de Westeringh (1957) in experiments on the uptake of indole-3-acetic acid by carrot disks and segments of pea stem recorded that for longer periods of treatment there was less than a 7-fold change in the rate of uptake over three units of pH. It could be advanced that in the initial phase of uptake this diversity in the reactions

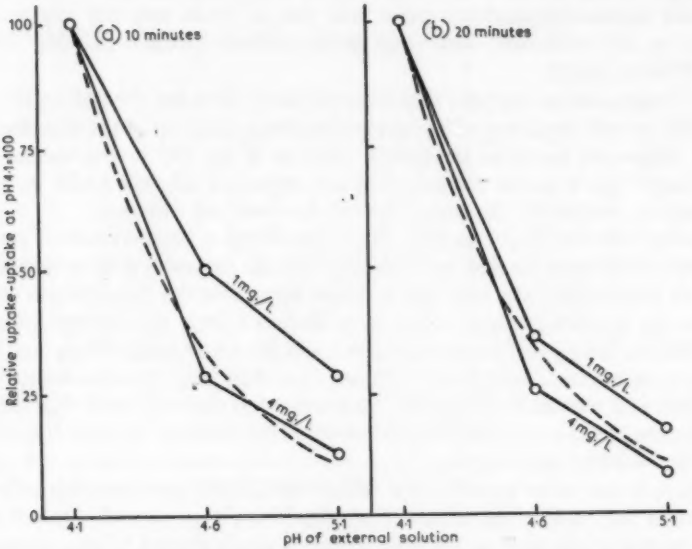

FIG. 14. Expt. 15. Uptake of 2:4-D in the first 10 (*a*) and 20 minutes (*b*), showing interrelation between the pH of the external solution, the concentration, and the corresponding concentration of undissociated molecules (broken line). For the purpose of comparison uptake and the proportion of undissociated molecules at pH 4·1 have both been equated to 100.

to pH is caused by differences either between compounds or species in the relative rates of entry of molecules and ions, together with the varying effects of the external pH on the internal pattern of cytoplasmic changes and hydrogen ion concentration.

In the foregoing discussion no distinction has been made between the processes of entry into the roots or the undersurfaces of the fronds. From previous investigations (Blackman and Robertson-Cunninghame, 1955) there is evidence that entry takes place through both types of tissue and it will be shown in the case of 2:3:5-triiodobenzoic acid that the pattern of entry and loss to the external solution has many features common to both roots and fronds. It can thus be concluded with reasonable assurance that the differences between *L. minor*, segments of *Avena* coleoptile and *Chlorella* are dependent on divergencies at cell level. From the viewpoint of initial uptake by *L. minor*, it would seem that either the period when physical processes are dominant is much shorter or that metabolic processes play a greater role from the start.

However, the major specific difference lies in the factors controlling the outward movement to the external solution. In this connexion it is more reasonable to suppose that there is a progressive reorientation of processes involving diffusion, adsorption, and chemical binding than that there has been a toxic disorganization since it is clear from Fig. 12 that with low concentrations and short periods of treatment the effects are reversible: indeed growth can be accelerated.

Lastly, the results of Expt. 7 (Fig. 4) are of very considerable significance. It has been established that the course of uptake is linked with the chemical structure of the growth regulator, which also determines its general physiological activity. The pattern of uptake is 'normal' for phenoxyacetic acid, which is accepted as an auxin of low activity, and that with the increasing physiological activity associated with the substitution of one or two chlorine atoms in the benzene ring, the tendency for the initial phase of uptake to be replaced by a phase of loss becomes more pronounced. This relationship between structure and these physiological properties is even more significant in the light of current research. Initial experiments (see Blackman, 1958) have established that the course of uptake of 2:4-D by the roots of species, which in agronomic practice are regarded as resistant, resembles that for phenoxyacetic acid while for susceptible species, the rate of loss to the external solution with time exceeds the rate of entry. Thus, it should now be possible to integrate in a single approach specific differences in chemical structure and cell characteristics in the analysis of the selective action of growth regulators.

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Observations on the Structure of the Zoospores of *Vaucheria*, II

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WITH 8 PLATES

Received 22 June 1958

SUMMARY

Sections of *Vaucheria* zoospores fixed at various stages have been examined in the electron microscope. Changes in the organization of the cytoplasm and the redistribution of its organelles are described and correlated with observations of living spores during their transformation into vegetative cells. In particular, proof is given that the disappearance of the cilia involves the withdrawal of the fibrillar axes of these organs into the cytoplasm and that the disappearance of the transparent outer zone of the motile spore involves the inward movement of special cytoplasmic vesicles which form a main component of this layer.

As a result of improved fixation new structural details are given, the most important of which concern the cytoplasmic matrix, the boundary membranes of the nuclei, the golgi bodies and their relation to mitochondria, and the internal structure of the chloroplasts. With the aid of serial sections it is shown that the internal laminae of the chloroplast are connected with each other in a special manner.

INTRODUCTION

IN a recent study of the motile zoospore of *Vaucheria* (Greenwood, Manton, and Clarke, 1957) the organization and structure were described with special attention to the ciliary equipment. The present objective has been to discover how the spore becomes reorganized and what happens to the cilia when, in the normal course of development, it ceases to swim and becomes a sessile vegetative cell.

Living spores are readily kept under observation during the few minutes occupied by this transformation but their densely green opacity and large size make them unrewarding objects for the more elaborate methods of visual microscopy and little has recently been added to the excellent accounts published in the nineteenth century by the pioneer observers. The literature was surveyed in the previous paper (loc. cit.) and reference need be made again only to the work of Thuret and of Strasburger both of whom, in describing the way the spores settle down, gave consideration to some of the problems that still confront the visual observer. In an early and admirably clear account Thuret (1843) noted that when the spore comes finally to rest the cilia first become motionless and are therefore separately visible; then there is a transient disturbance of the surface of the spore during which the cilia vanish completely, but whether these organs were lost by decomposition or by absorption he could not say. He believed the clear peripheral border of the motile spore to be a form of covering membrane. The gradual disappearance of this border

and the outward movement of the green contents as the spore settles down and rounds off suggested to him that during this process the covering layer becomes thinner, perhaps by some process of partial absorption. Strasburger (1880), writing almost forty years later, could add few new observations except for details of the way in which the cilia appear to pass from view, although with a more complete knowledge of the structure of cells in general and additional evidence from stained microtome sections of the *Vaucheria* spore itself he gave a fuller and rather different interpretation of what he was able to see. His final concept of the newly emerged swarmer as a naked cell, the transparent outer zone of which is a special layer of the protoplasm devoid of chloroplasts but containing all the nuclei, is now generally accepted and has recently been given confirmation and additional detail by the study of electron micrographs (Greenwood *et al.*, 1957). He believed, however, that before the close of its swimming phase the spore becomes invested with a thin cellulose wall through holes in which the still active cilia project, and he described the wrinkling of the spore surface which accompanies the loss of the cilia as undulations in this wall. The shortening of the cilia was said to be associated with the formation of a small vesicle at the tip of each, which gradually enlarged as the cilium shortened until, after a few seconds, the cilia disappeared, leaving the spore surface quite smooth. He concluded that the cilia are withdrawn into the cell, passing in via the holes through which they projected. After the loss of the cilia he was able to see a cell wall increasing rapidly in thickness and he equated the decreasing width of the clear transparent border within it with a submergence of the peripheral protoplasm as the chloroplasts moved outwards and the nuclei moved inwards.

From these observations, in spite of uncertainty in details it has long been obvious that the surface of the *Vaucheria* spore must undergo very fundamental and rapid changes during the brief period which separates the swimming phase from the succeeding phase of normal vegetative growth. The aims of the present investigation have been to investigate these and other more internal changes using the powerful tools of thin sections and electron microscopy which are now available. Since, however, it is essential to become familiar with details of behaviour of the material which can be seen in life before electron microscopy can be effectively applied, a brief account of personal observations made with the light microscope will precede the discussion of sections. This is the more necessary in that we already know from previous work (Greenwood *et al.*, 1957) that some of Strasburger's concepts, notably that of the early presence of a thin cellulose wall with holes for the cilia, are errors of interpretation of genuine appearances the nature of which can now be better understood.

MATERIALS AND METHODS

Wild material was preferred for the reasons given previously (Greenwood *et al.*, 1957). It was collected in spring from the same site as before though at this season sex organs are normally absent and specific identification of the zoospores is therefore difficult and of necessity indirect. On this occasion, with

the co-operation of Dr. T. Christensen of Copenhagen, some of our vegetative material has been brought into cultivation and accurate determinations made. A gathering of filaments sent by post to Copenhagen in March 1957 developed sexual plants of three species, namely, *V. sessilis* (Vauch.) D.C., *V. hamata* sensu Goetz s. l, and *V. terrestris* sensu Goetz. Since zoospore formation does not normally occur in the last two species but is copious in the first it seems almost certain that the material used for this and the previous study was in fact *V. sessilis*. This, it may be noted, is the species described by Strasburger though Thuret, encountering a similar situation to our own, left the identification of his spores uncertain.

The spores were caught whilst still motile; a few were fixed at once, but the others were kept in water until some minutes after swimming had ceased. Several spores (e.g. V58, Fig. 2), were kept individually under continuous observation and fixed at known stages; others in groups of up to twenty were collected quickly, allowed to settle and fixed at known times after capture but without recording the moment at which each ceased to swim.

Fixation, for 1 to 2 hours, was in cold (4–8° C.) 1 per cent. osmium tetroxide buffered with acetate veronal at pH 7·0 to 7·2. A prior addition of calcium chloride (in varying amounts between 0·2 and 1 per cent.) which was made to the fixative for some of the spores, had the advantage that in the motile condition the covering membrane of a spore and its cilia was retained more completely (Fig. 9). Internally the addition of this reagent gave a very coarse pattern of fixation with loss of fine detail in most of the protoplasmic structures although the specific nature of this effect on one category of vesicle (Pl. VIII) has provided important new facts.

Washing and dehydration up to 95 per cent. alcohol were carried out in covered watchglasses at about 8° C. but the completion of dehydration and graded transfer to methacrylate monomer were at room temperature. In the case of spores handled in batches the final methacrylate mixture was raised to about 40° C. to avoid contamination with water vapour from the atmosphere whilst the spores were being separately placed in individual gelatin capsules for embedding. In other details the preparation of embedded material for electron microscopy followed the routine previously described.

Satisfactory photographs of optical sections of the embedded spores can be obtained from the uncut blocks especially if these are submerged in glycerine with the spore uppermost (Figs. 1 and 3). After sectioning, an extensive survey at low magnifications was carried out with the old Philips electron microscope in the Leeds Botany Department (Figs. 2, 4, 23, and 25). The other electron micrographs were taken in microscopes of greater resolving power, namely a more modern Philips instrument (Figs. 6, 10, and 11) and a Siemens Elmiskop I (all the remaining figures).

OBSERVATIONS WITH THE LIGHT MICROSCOPE

The behaviour of the spores in life. The free-swimming phase when watched alive is brief (5–10 minutes usually) and intermittent. The spores frequently

rest or revolve slowly in the surface film or they may pause at the bottom of the dish or elsewhere. During these temporary halts the cilia continue to beat, though more slowly, and a spore may often be encouraged to swim again by a gentle touch or an agitation of the water, a circumstance which aids the rapid discovery and capture of ciliated spores. Periods of immobility gradually become more frequent and more prolonged until locomotion finally ceases. Under high magnifications it can then be seen that the permanent loss of motility is initiated by a rather abrupt cessation of the rhythmic beat of the cilia which, for a few seconds, become separately visible extended straight outwards, held normally or at some smaller angle to the cell surface, moving only slightly or not at all.

The spore now undergoes rapid change, and in less than half a minute at room temperature the very pronounced undulations of the surface that accompany the shortening and loss of the cilia give place to a smooth and evenly rounded external contour although the attainment of the final almost spherical form may be delayed until a little later. Reorganization is not yet complete and some minutes elapse before the brightly transparent border has narrowed to vanishing point by which time the cell wall has become clearly evident. Measurements of spherical cells (not quoted here in detail), show that a process of enlargement has begun which may continue for several hours until the spores are so transparent that the cytoplasm can be seen to have become only a thin layer surrounding a much enlarged and centrally placed main vacuole. Before the clear border has gone a spore can be plastically deformed by gentle pressure and after the loss of the cilia its surface is at first apparently sticky, the spore adhering readily to other objects upon which, if roughly pulled away, it may leave a portion of its peripheral substance. If the damage is slight, repair appears to take place and such spores may attain spherical form and become paler in colour in the normal way. Once a wall is clearly visible the spore is less plastic and the surface markedly less sticky.

The process of development is very temperature-dependent and is probably affected also by light. In diffuse daylight at about 16–20° C. a clearly defined wall and some degree of expansion of the spore may be evident within 10 minutes of settling down, but at temperatures between 0° C. and 8° C. the spore may remain for half an hour or longer with the colourless cytoplasm only partially eliminated from the periphery and without a well-marked wall.

Visual observations on the embedded spores. Embedded spores are sufficiently transparent for the limits of the cytoplasm and the main vacuole to be observed in optical section. Fig. 8 of a motile spore, has been reproduced from the previous paper (Plate I, Greenwood *et al.*, 1957), although at reduced magnification, to facilitate comparison with the two transient conditions of settled spores shown in Figs. 1 and 3.

The spore in Fig. 1, recently denuded of cilia, was in life smooth and rounded although the clear peripheral cytoplasm had not yet disappeared. The vacuole, which was anterior in the ovoid motile spore (Fig. 8), is now central, altered little in size but with an irregular boundary so that the cytoplasm,

which accounts for the main bulk of the cell, is uneven in thickness. Spores like this are extremely soft after osmic fixation and some surface damage can hardly be avoided, but even at torn places there is no sign of a wall separate enough to be parted from the underlying cytoplasm.

A later stage is shown in Fig. 3 and the well-developed wall, though sufficiently stout to prevent the surface damage to which earlier stages are prone, is made evident by the way it has become ruptured and partially lifted from the cytoplasm; an artifact that becomes visible during infiltration with methacrylate. The spore has expanded considerably; the vacuole, which has now a smooth boundary, more or less concentric with the exterior of the spore, is larger and the cytoplasm forms a uniform but thinner layer which in life has lost its shining peripheral zone.

OBSERVATIONS WITH THE ELECTRON MICROSCOPE

General organization of the spore. The gross features of organization in settled spores are displayed in the low-power survey fields of Figs. 2 and 4 which may be compared with the section of a motile spore shown in Fig. 31 of the previous paper.

Fig. 2 is from a spore kept under observation at 10° C. until eight minutes after swimming had ceased. No cilia are visible externally but the nuclei (most easily recognized as such when sections include nucleoli) remain close to the external surface though no longer in contact with it. The peripheral cytoplasm, from which chloroplasts are still excluded, retains at this stage the vesicular organization found in the motile spore. In the cytoplasm bordering the vacuole the characteristic 'vesicles with contents' are also still present (Fig. 2, left) though less tightly packed together. Chloroplasts and fat bodies are conspicuous but it must be noted that the slight flattening of both and the parallel banding of the latter are compression artifacts introduced during sectioning.

Fig. 4 is from one of a batch of spores kept at 10° C. for 20 minutes before fixation. The more even vacuolar boundary and shallower protoplasm are obvious and a small part of the cell wall can be seen lifted free from the surface, at the extreme right-hand side of the figure. The outer cytoplasm has lost its special organization; the nuclei, mostly in groups, have become more deeply immersed in the cytoplasm whilst chloroplasts, fat bodies, and mitochondria are found at all levels between the tonoplast and the cell surface.

The internal cilia. At higher magnifications substantial remnants of the ciliary apparatus are at once evident inside the spore, so numerous and so distinctive in morphology that they are land-marks among other less well characterized cytoplasmic structures. They will therefore be described first although a fuller appreciation of their location in the cytoplasm will follow when more details of the latter have been provided.

The peculiarly intimate relationship between the nuclei and the ciliary bases (see Pls. VII and X to XV in the previous paper) is maintained even when the cilia have been withdrawn. Longitudinally cut ciliary bases inside the spores are found almost invariably to be located at one end of a nucleus, in

line with its long axis, and, at least in early stages, inclined towards the cell surface. Fig. 5 shows a group of three nuclei, two with prominent nucleoli and one without, and attached to the latter is an easily recognizable tangential L.S. of a cilium and its base (lowermost arrow). A dark object similarly placed close to the upper large nucleus is a very oblique section of a similarly attached base. Sections showing the longitudinal view of both of the ciliary bases related to a nucleus are infrequent though some (not reproduced) have been found.

Transverse sections of cilia with their very characteristic 9+2 arrangement of fibres are very numerous in the outer cytoplasm between the nuclei and the cell surface. Several, mostly in pairs, are indicated by the remaining arrows in Figs. 5 and 22 with two pairs included at a higher magnification in Fig. 7.

In all these profiles a striking feature is the absence of a ciliary membrane (see especially Figs. 6 and 7) although before withdrawal the cilia are covered by a thin skin continuous with the plasma membrane of the cell surface (Fig. 9). In spite of this lack the cytoplasmic granules (see p. 62) do not invade the interior of the ciliary axis which, when cut longitudinally is remarkably uniform in diameter and straight over considerable distances. In two cases a straight run of 10 μ was directly observed, a distance which is scarcely less than the whole length of a cilium as measured on the motile spore (loc. cit.). All this suggests not only that the ciliary axis as a whole is relatively rigid but that the apparent spaces between the fibres are occupied by some material component which also prevents displacement of the central strands.

The structure of the cytoplasm. The cytoplasm is separated from the main vacuole by a membrane as described for the motile spore (Greenwood *et al.*, 1957) but the outer limit of the cytoplasm, which is a site of vigorous change, cannot be so easily characterized and is, moreover, difficult to preserve intact. In the condition of Fig. 2 there is evidence of a simple boundary, appearing rather thicker than that which covered the motile spore, but apparently in direct contact with the underlying elements of the cytoplasm. It is seen in Fig. 10 as an even line in the surface in conformity with the smooth visual contour of the spore at this stage. No recognizable parts of residual ciliary membranes can be distinguished outside the cell with certainty although a certain amount of debris of membranous character is always present on the outside of the settled spore (Figs. 10–11). Further details about the surface boundary will be reserved for a later communication.

Internally much of the characteristic texture of the cytoplasm visible in the sections is given by the number and diversity of its vesicular components, particularly the large ones (from about 0·2 to several μ in diameter) which account for a great part of its volume. Although the vesicles all have, as the common definitive feature, an essentially closed boundary surface formed by a thin and simple plasma membrane, some appear empty whereas others have contents of several distinctive types. The existence of a number of separate categories of vesicular components, some of which are of special structural significance, is made additionally evident by the systematic grouping of different sizes and

types of vesicle in particular regions of the cytoplasm and by the rearrangements that these undergo as the spore passes through its developmental stages.

In the recently settled spores (Fig. 2) the peripheral layers of the cytoplasm are conspicuous because of the relatively uniform size, empty appearance and close packing of the vesicles which form its main component. These, by their mutual contact, provide a framework within the interstices of which smaller vesicles and other cytoplasmic constituents are located. Below this zone the cytoplasm appears to be more loosely organized, the plastids and other organelles are numerous and the vesicles more varied in size and in contents. The largest vesicles tend to be close to the vacuolar boundary, a region of the cytoplasm which is given character also by the special category of 'vesicles with contents' mentioned previously (p. 59).

During the reorganization of the zoospore on conversion into a spherical walled cell the innermost 'vesicles with contents' remain close to the vacuolar boundary. On the other hand, special interest attaches to the fate of the empty looking vesicles of the peripheral cytoplasm which rapidly cease to be evident in that position. They remain visible for some time after the withdrawal of the cilia during the stage in which the spore remains plastically deformable but, without special treatment, they cannot later be distinguished from other empty looking vesicles always present at all levels in the cytoplasm (e.g. see Pls. II, V, and VI).

The addition of calcium chloride to the fixative (especially in the highest concentration used of about 1 per cent.), whilst it produces a loss of fine detail in most structures has a specific effect on the contents of the peripheral vesicles which distinguishes them as being of special nature. In the low-power view of a section from the anterior end of a motile spore treated in this way (Fig. 23) each of the peripheral vesicles contains an opaque oval or reniform body, smoothly bounded and set in a particularly transparent space; these conspicuous bodies (seen at higher magnification in Fig. 24), are no doubt the coagulated and contracted contents of the vesicles heavily loaded with osmium. At this stage only a few similar vesicles are present in the more internal layers of the cytoplasm where they are readily distinguishable. On the other hand, in settled spores which have acquired a cell wall (Fig. 25), vesicles with identical dark contents are generally distributed and numerous at all levels of the cytoplasm and it cannot be doubted that their disappearance from the periphery is due to physical displacement rather than to destruction or transformation.

Other categories of vesicle are present, recognizable by contents of specific form but of unknown nature or function, among which may be mentioned the clearly defined and well-dispersed hollow spheroids seen in the vesicle in the bottom right of Fig. 7 and the more organized vermiform or rod-like contents of the vesicle indicated by an arrow in Fig. 16.

The cytoplasm also possesses a continuous matrix between the vesicles and other protoplasmic structures. This matrix is characterized by the presence of varying numbers of fine granules. Fig. 6 shows this granular material at

various levels in the spore and a selected area in this field is reproduced at a higher magnification in Fig. 12. The characteristic granules are extremely electron dense. They are for the most part somewhat isodiametric in shape though not spherical and about 150 Å in diameter with a few up to 300 Å. They agree in range of shape and size with the granular component of animal cytoplasm described by Palade (Palade, 1955), an additional characteristic feature of which was a tendency to occur in chains or rosettes. A similar arrangement is also demonstrable in *Vaucheria* as may be seen in many parts of Fig. 12. When arranged in chains or rosettes the granules tend to be equidistant and even when more randomly dispersed they rarely if ever touch. Occasional appearances as of granules in lateral contact are likely in most cases to be due to overlapping of particles occupying different levels in the thickness of a section. Rarely, granules appear to be fused to one surface of a membrane (Fig. 13, arrow), in which case the spacing is comparable to that of the granules in chains though the shapes are slightly different, the attached granules being oblong with the long axis perpendicular to the membrane. More than one category of granule may be involved but it is noteworthy that those arranged in chains may also tend to depart somewhat from isodiametric form in this manner although there is no reason to believe that the chains are also attached to membranes.

The nuclei. It is not easy to determine the exact shape of the nuclei after contact with the cell surface is lost though they are certainly more nearly isodiametric (cf. Fig. 4, &c.) than in the motile spore (1957, *loc. cit.*). Each nucleus is enclosed by two membranes which are in contact only at isolated places, and the 'perinuclear space' (Watson, 1956) between them is seen as a system of clear areas around all but the most tangential of profiles; each nucleus in Fig. 5, for example, is recognizable by this feature. The inner membrane is in contact with the nuclear contents whereas the outer is an internal boundary of the cytoplasmic matrix. The two membranes are, however, not completely separate, the places at which the perinuclear space is obliterated (arrows on Figs. 14 and 15) being more definitely organized than mere zones of contact. These junctions, or pores, are seen as circular profiles in sections cut at grazing incidence to the nuclear surface. Such a section lies in the centre of Fig. 15 between the deeply cut nucleus (upper left) and the pair of ciliary bases (lower right) and it bears five annuli of the size expected (about 750 Å). In sections cut normally to the nuclear surface (arrow in Fig. 15), the outer and inner membranes can be seen to be confluent at the edges of the annuli. In thin sections the 'pores' appear sometimes to be open although there is no evidence for an intermingling of nuclear and cytoplasmic material. Often, however, and in thicker sections usually (Figs. 14 and 15), a region of greater electron density crosses the 'pores' but it is diffuse, unlike a single or double membrane in section, and may be no more than a view of the opposite rim included in the thickness of the section.

With regard to the nuclear contents there is little to add to our previous account except to draw attention to the apparently granular nature of the some-

what stellate nucleoli, the extensions from which can sometimes be traced to the nuclear surface (Fig. 15).

Mitochondria and associated structures. At the stage of Fig. 4 the mitochondria are rather uniformly distributed in the cytoplasm; they may lie against nuclei or more frequently (Figs. 19 and 21) against chloroplasts, but there is little regularity of association or obvious structural connexion in these juxtapositions, metabolically significant though they may perhaps be. A most intimate relationship is, however, regularly found with another organized structure of the cytoplasm, examples of which are shown in Pl. V and, incidentally, in Pl. VII.

Attention is directed first to Fig. 16 in which each of two mitochondria (recognizable by the internal microvilli) has closely applied to its right-hand surface a group of vesicles and parallel membranes. These possess the more obvious morphological features characteristic of golgi bodies (or dictyosomes) in other organisms, namely a central group of large, flattened membrane-bounded spaces surrounded by smaller more rounded vesicles, except that here an additional array of flattened membranes is present against the mitochondrial surface. A section cut in the right plane to show this clearly is given in Fig. 18, the upper half of which includes part of a mitochondrion with a golgi body in close contact. The double boundary of the mitochondrion is plainly visible and immediately beside and parallel with it is what appears to be a large flattened vesicle. Below this is a zone of small rounded vesicles followed by the conspicuous array of paired membranes which form the central feature of the golgi area. These membranes form the closely approximated boundaries of 2 to 4 narrow spaces, flattened in a plane parallel to the mitochondrial surface but expanded often at their ends into rounded cavities similar to the small vesicles which surround them and crowd the neighbouring cytoplasm. The same pattern of organization is exposed in all approximately median sections passing through both attached organs and the flattened spaces are therefore necessarily interpreted not as tubules but as parts of sacs.

Golgi bodies do not seem to occur unattached to mitochondria; each golgi body is shaped like a flake or pellet pressed firmly and centrally against the attendant mitochondrion which is usually distinctly flattened at the contact surface. Sections such as that of Fig. 17 where the flattened sacs are less in evidence and in which a greater proportion of the mitochondrial microvilli are cut transversely are useful for showing the vesicular composition of a golgi body near its edge. There is no common enclosing envelope or other barrier interposed between the vesicles and the cytoplasm, the granules of which, although able to enter the spaces between the outermost small vesicles (Fig. 18), are quite excluded from the central region. This fact, and the constancy of the configuration described above, indicates that the membranes of the vesicles and sacs in the centre of the golgi area are tightly pressed into contact if not actually adherent or fused to each other. Nothing is known about the contents of the sacs.

The chloroplasts. The majority of the chloroplasts in settled zoospores are

shaped like biconvex lenses (3–5 μ in diameter), subcircular in face view and elliptical in cross-section. As the rearrangement of the contents of a spore continues the plastids tend to an increasing degree to become orientated parallel to the cell surface (compare Figs. 2 and 4). Sections of spherical spores may include fields like that of Fig. 19 almost every chloroplast in which has been cut perpendicularly to its internal laminae which occupy a system of planes parallel, predominantly, to the faces of the plastid.

The comparatively simple construction of the chloroplasts, which lack specially organized pyrenoids, eyespots, or grana is well displayed in median cross-sections such as the larger profiles visible in Fig. 19. The laminae (each of which is itself compound) are few in number, usually between 11 and 15, and appear as dark bands separated by layers of the less opaque ground substance or matrix of the chloroplast. Most of the laminae terminate short of the ends of the profiles (see Figs. 20 and 21 for higher magnifications), appearing to end blindly in the matrix or by confluence in some way with another lamina (see p. 65). Longer ones may curve round the contour of the chloroplast, lying close to its boundary and thus enclosing some or all of the shorter ones. In a proportion of cases (as in some of those in Fig. 19) a lenticular expansion of the matrix, usually more or less centrally placed, divides the laminae into two rather loosely constituted groups.

The ground substance (chloroplast matrix) between the laminae has numerous granules, rather smaller than those of the cytoplasm and not found arranged in chains (a comparison made most readily in Fig. 20). There are other opaque inclusion bodies also to be found in the matrix, two types being visible in typical form and distribution in many of the plastids in Fig. 19. The larger bodies (up to 4,000 Å across) have a diffuse outline (Fig. 20) and are usually found in the matrix of the central zone when this is visible (Fig. 19 in several places) though other positions can occur (Figs. 20 and 22). The smaller bodies (up to 1,000 Å) are more opaque and more sharply delimited from the matrix. They are often more numerous and their position (e.g. two in the chloroplast on the left of the μ mark in Fig. 19) is usually between the laminae rather than in the centre of the plastid though they are sometimes present there also.

That the laminae themselves are always compound is most obvious where the knife has passed along their length as in Fig. 20 and in the main plastid of Fig. 21. In Fig. 19 and in the portion of a plastid in the upper corner of Fig. 21 the direction of knife compression has been such (across the laminae) as to hold the component layers in contact rather than to separate them. Each lamina (which may be no more than about 200 Å–300 Å in thickness) contains a pair of lamellae which appear as two separate dense lines each about 75 Å in thickness. Although each of these lamellae is itself almost certainly compound, no instance of more than two clearly defined constituent lamellae of this thickness has been found, the frequent appearance of four components in each lamina (clearly seen in many places in Fig. 20) being that of two internal lamellae flanked by the thinner boundaries of the surrounding matrix.

The firm construction of these lamellae themselves is attested by the manner in which they are thrown into folds and separated by clear spaces from each other or from the matrix when the knife has passed as in Fig. 20.

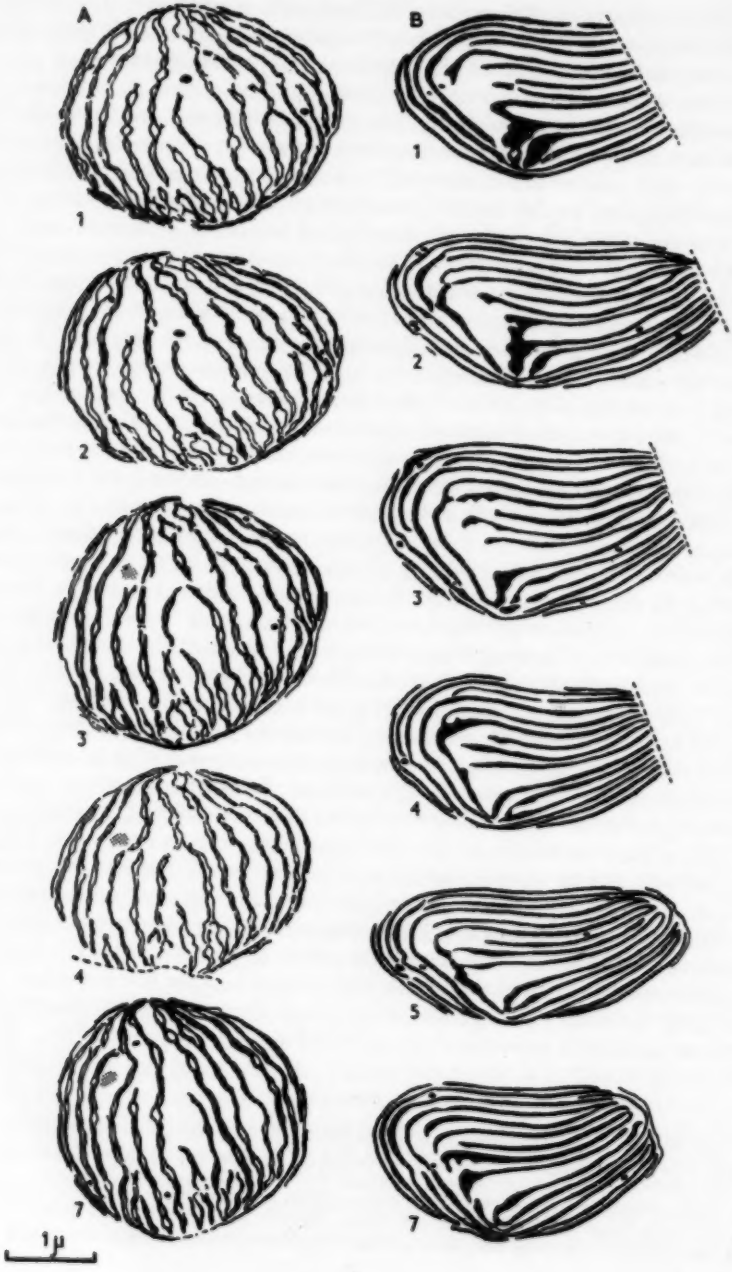
A detailed three-dimensional reconstruction of the system of laminae in a chloroplast (which is in preparation) requires fuller treatment than can be given here, but because single sections convey the quite erroneous impression that the small number of laminae are largely separate from each other with few points of junction, Fig. 26 has been included. This illustrates diagrammatically the laminae of two plastids in sections selected from a series of seven, each of which passed through both chloroplasts. Portions of two of these sections have been reproduced as Figs. 21 and 22. Plastid A, Diagram 1, shows a number of long laminae passing completely across it with some shorter ones between them which start at an edge and end abruptly nearer the centre or, in a few cases, appear to fuse with an adjacent lamina. Examination of consecutive sections gives information on the origin of these 'fusions' and in every analysable case a fusion involves a long lamina and a short one which, in other sections of the series, can be seen to end freely in the matrix. Such fusions occur in a very regular manner, each short lamina becoming confluent with a longer neighbour, which in this particular plastid is always on the right, forming a ⅄-shaped junction. Almost at once, usually in the immediately adjacent section, the right-hand limb of the ⅄ becomes detached to form a new short lamina. At these places the discontinuity, where a segment from one lamina passes into the plane of an adjacent one with a reciprocal discontinuity, establishes a connexion between otherwise separate regions of the matrix, and inclusion bodies of both types are often, although not exclusively, located in or near such connexions. In plastid B, Diagram 2, several series of discontinuities and exchanges between adjacent pairs of laminae can be followed, notably at the left-hand ends of the profiles where several inclusion bodies of the smaller type are also involved in the structure. An estimate of the thickness of the sections shows that these seven sections comprise less than one-eighth of the diameter of the chloroplast[1] and demonstrates (and similar results with longer series have confirmed), that far from being a simple array of parallel sheets the laminae are abundantly fenestrated and cross linked so as to form a unified structure.

At the periphery of the plastid the superficial laminae approach the limiting surfaces of the plastid so closely and the matrix layers become so thin that the separate contributions of chloroplast and cytoplasm to the boundary have not been unequivocally distinguished. Particularly clear views of the margin of a chloroplast may be seen at several places in Fig. 20.

DISCUSSION

As will be clearly evident to anyone familiar with the previous paper some of the new observations recorded above depend not so much upon the develop-

[1] The sections were probably about 0·025 μ thick, and allowing for an equal thickness destroyed between each (Williams and Kallman, 1955) the total depth of chloroplast involved in the series of 7 is no more than about $\frac{1}{3}$ μ, less than one-eighth of the diameter of the plastid.


A
B
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mental processes studied as upon the fact that at these stages good fixation is more easily achieved than was the case with the motile spore. The improved quality of fixation has contributed new facts regarding several of the cell components, the more important of which are: the nuclear membranes, perinuclear spaces and 'pores'; the Palade type particles in the cytoplasmic ground substance; the golgi bodies and their relation to the mitochondria; certain structural details within the chloroplasts.

The more important general conclusions which can be drawn from these new structural observations would appear to be as follows:

(*a*) The chloroplast data are of considerable comparative interest on the one hand in amplifying previous knowledge of the chloroplasts of the heterokont groups of the algae (e.g. Phaeophyta; Leyon and Von Wettstein, 1954; Manton, 1957: Chrysophyta; Manton, 1955; Parke, Manton, and Clarke, 1958), and, on the other hand, in amplifying the other types of evidence affecting the taxonomic position of *Vaucheria*. The recent removal of this alga from the Chlorophyceae to the Xanthophyceae is as strongly supported by the structure of the plastid as it was by our own observations on the flagella. The analysis of serial cross-sections of chloroplasts has shown that in *Vaucheria* the simple patterns made by the laminae visible in each individual profile are to be interpreted as sectional views of a complex laminated cage rather than of a small number of separate parallel sheets.

(*b*) The presence of Palade type granules in the cytoplasm of the alga has the additional interest that the general distribution of the granules in the interstices between the vesicles and organelles demonstrate more clearly than we were previously able to do the existence of a continuous phase of the cytoplasmic ground substance.

(*c*) The intimate physical union between the mitochondria and golgi bodies is an unusual feature which may perhaps be functionally related to the extensive degree of relative movement between the different protoplasmic structures imposed by the processes of development that the coenocyte undergoes.

With regard to the developmental processes studied, the more important observations include: the demonstration of the cilia within the spore after their disappearance from the exterior; the establishment of a method for distinguishing one special category of vesicle; the various observations on the movements of the larger organelles.

All these observations can be correlated readily with the changes seen under the light microscope. Since the withdrawal of the cilia cannot, however, be effectively studied visually in life, this is the first conclusive evidence of their fate. Concerning the peripheral vesicles, the electron microscope has demon-

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FIG. 26. Diagrams of two chloroplasts (Series A and Series B) made from sections selected from a consecutive set of seven, and numbered accordingly. To show, in each chloroplast, relationships between the parts of the system of internal laminae and the locations of inclusion bodies of two types, the larger type shown stippled and the smaller solid. Other details are omitted. Representative fields from two of these sections, which include chloroplast A and part of B, are reproduced as Figs. 21 and 22.

strated their special contents and the major structural role which they must have in the outer cytoplasm of the naked protoplast. This information suggests that further investigation of their chemical and physical nature, involving other and more direct methods, may reveal the extent to which they confer on this limiting zone of cytoplasm the many special properties, such as plasticity and capacity for self-repair, which it appears to possess in life.

ACKNOWLEDGEMENTS

The writer is greatly indebted to Dr. T. Christensen for examining a representative sample of the living material and for his kindness in allowing the results to be reported here. The visual photographs (Figs. 1 and 3) were made by Mr. B. Clarke who also gave much appreciated assistance in the preparation of the plates. The high resolution microscopy was made possible only with facilities provided by Messrs. Philips during a visit to Eindhoven in July 1956, and by Messrs. Wykeham at the Aeon Laboratories, Egham, on several occasions. The very helpful co-operation of the staff at the two laboratories concerned is sincerely acknowledged.

The author is particularly grateful for the encouragement and advice of Professor I. Manton throughout the whole of the work.

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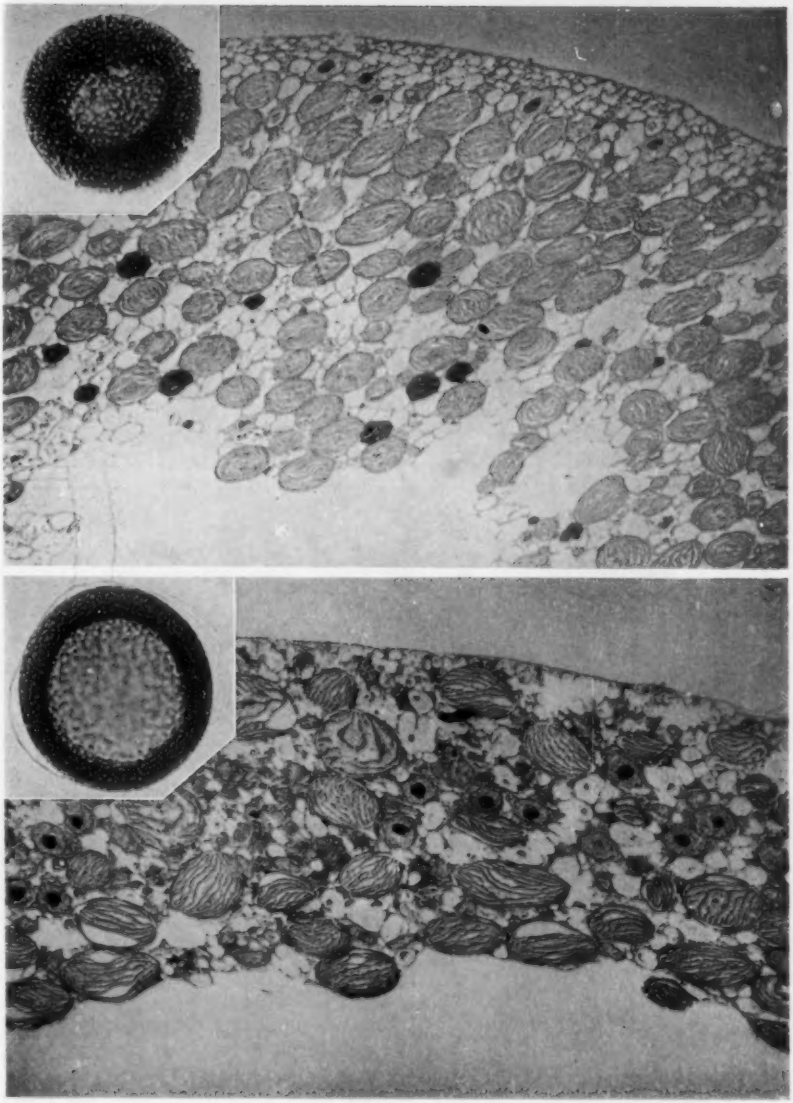

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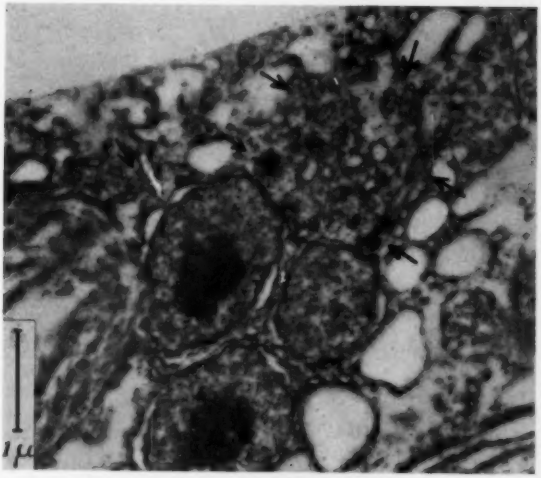
PLATE I

FIGS. 1–2. Two zoospores of Vaucheria at an early stage of rounding up; the cilia withdrawn but the nuclei and peripheral vesicles still near the surface. Fig. 1 an optical section (light microscope) of an intact spore embedded in methacrylate, ×200. Fig. 2 an actual section (electron microscope), ×4,500.

FIGS. 3–4. As Figs. 1–2 but a later stage with detectable cell walls, nuclei deeper in cytoplasm and peripheral vesicles dispersed.



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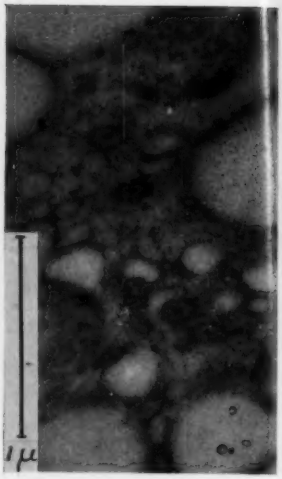

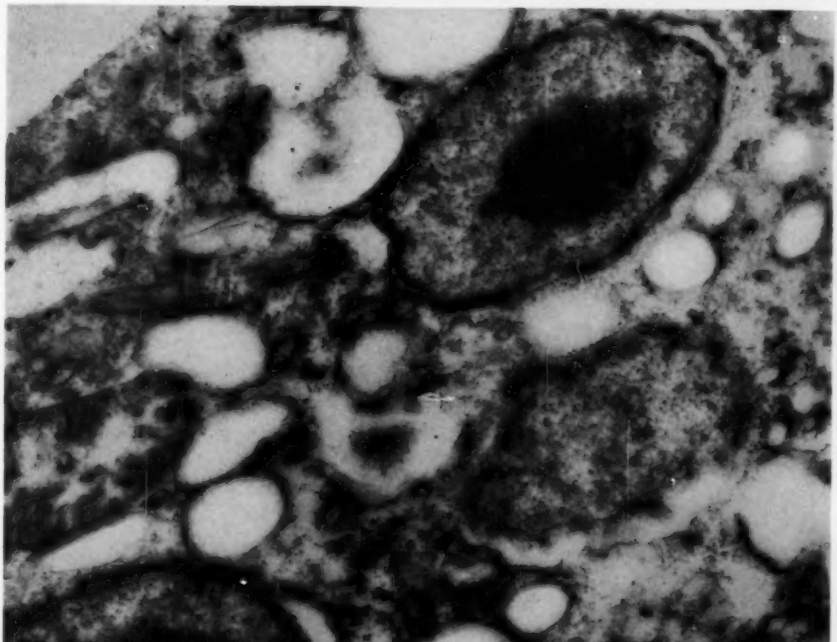

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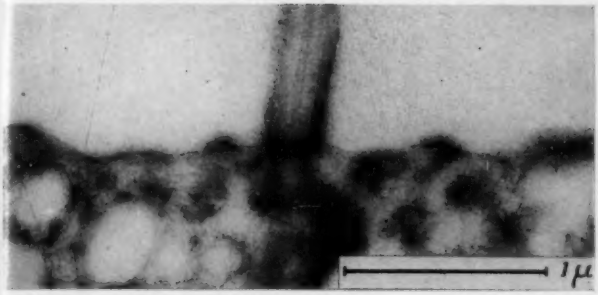
PLATE II



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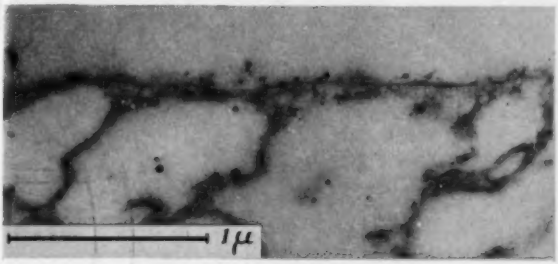

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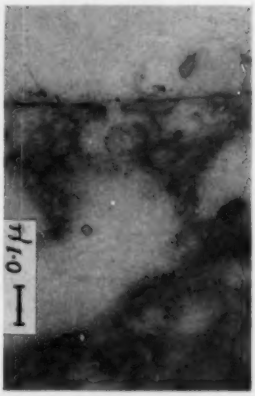

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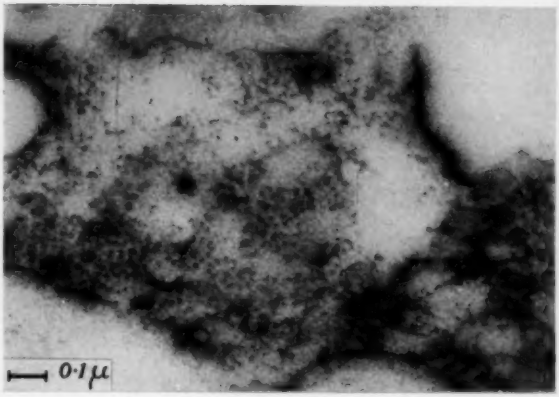

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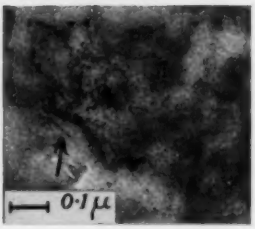

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PLATE III

FIGS. 5–6. Two parts of a section near the surface of the spore of Fig. 4 to show nuclei, associated ciliary bases, and cilia. Fig. 5, ×15,000 (GS7/11). Fig. 6, ×30,000 (GS6/20).

FIG. 7. Two pairs of cilia within the cytoplasm, from the spore of Fig. 2, ×30,000 (E396/18).

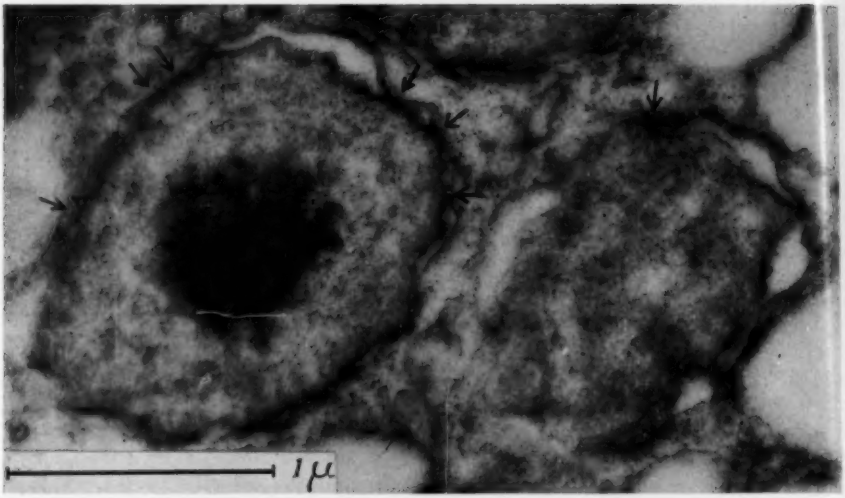
FIG. 8. Optical section through a motile spore, ×200 (reduced from Greenwood *et al.* 1957).

FIG. 9. Surface of a motile spore fixed in presence of $CaCl_2$ showing continuity of covering membrane of spore and cilium, ×30,000 (GS9/18).

FIGS. 10–11. Surface of a recently settled spore (cf. Fig. 2). Fig. 10 ×30,000 (E396/5). Fig. 11 ×60,000 (E396/10).

FIG. 12. Part of Fig. 6 to show arrangement of cytoplasmic granules, ×60,000.

FIG. 13. Part of Fig. 22 showing cytoplasmic granules associated with a membrane, ×60,000.



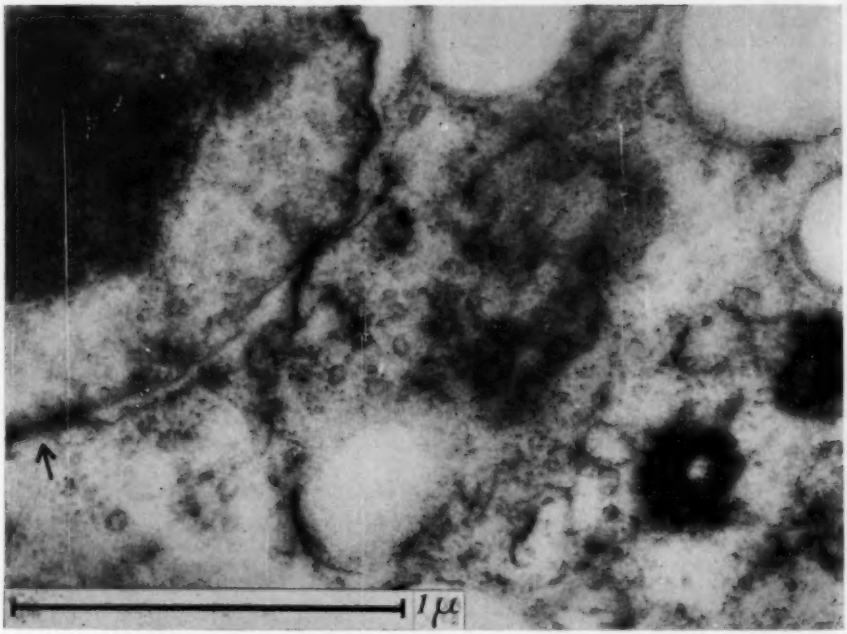
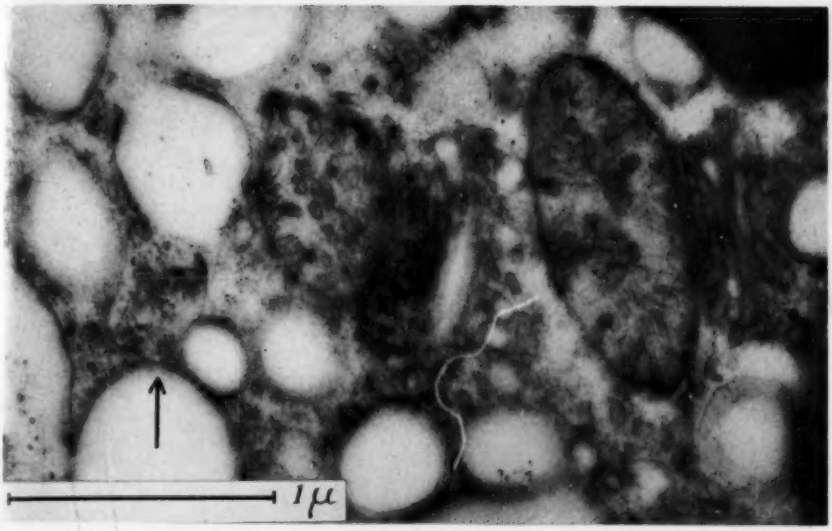


PLATE IV

FIGS. 14–15. Details of nuclear membrane from the spore of Fig. 4. Arrows in both Figs. point to 'pores' in radial section: for further description see text p. 62. Fig. 14, ×40,000: Fig. 15, ×60,000 (both GS6/24).



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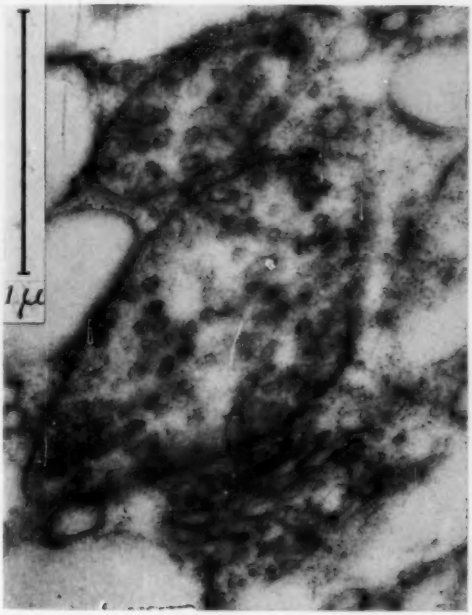

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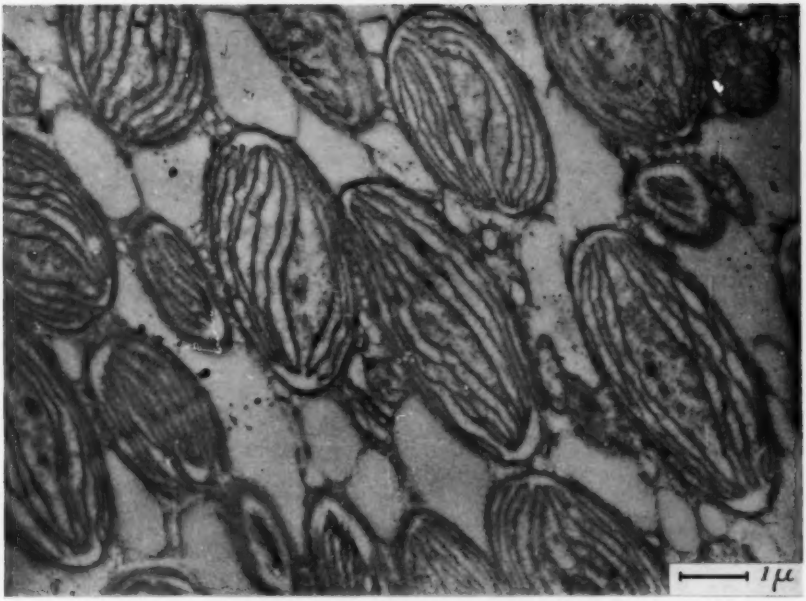
PLATE V

FIGS. 16–17. Cytoplasm of the spore of Fig. 4 with profiles of four mitochondria, three of which show accompanying golgi bodies, ×40,000 (GS7/3 and 6/22).

FIG. 18. A thinner section from the spore of Fig. 20 to show details of a golgi body and its contact zone with a mitochondrion, ×*c.* 50,000 (GS22/21).



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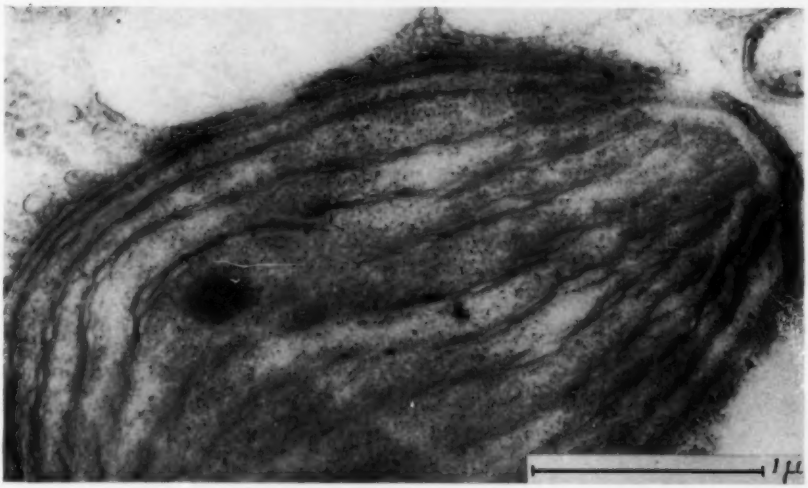

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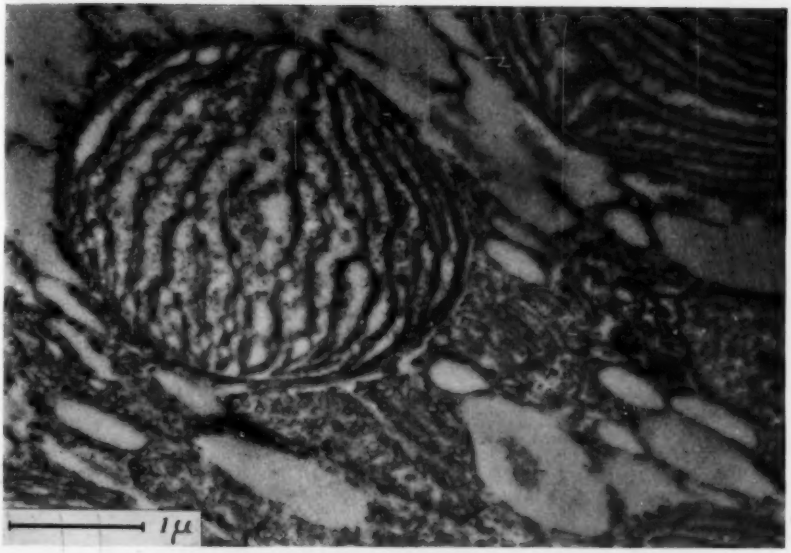
PLATE VI

FIG. 19. A group of chloroplasts from spore of Fig. 2, showing internal system of laminae and inclusion bodies of two types, × 15,000 (E396/31).

FIG. 20. Part of a chloroplast showing the components of the main laminae and an inclusion body of the larger type. The finely granular matrix material may be compared with that of the cytoplasmic matrix, × 35,000 (GS22/15).



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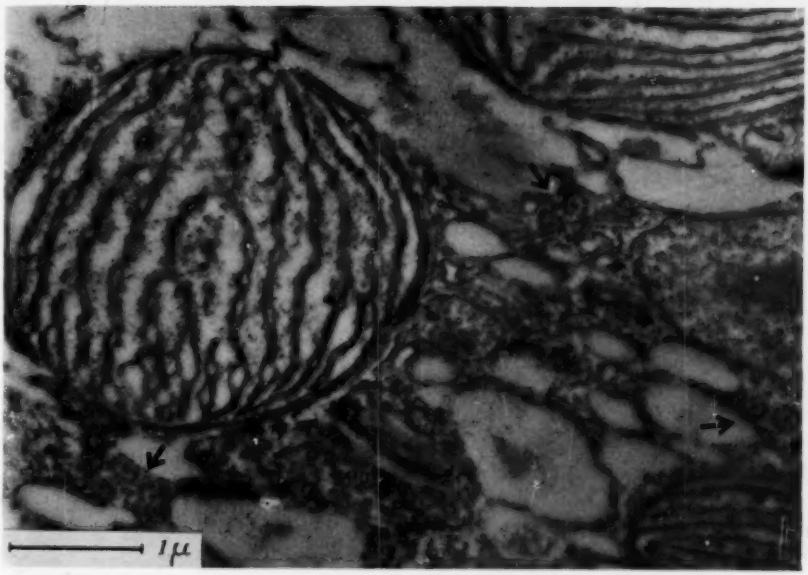

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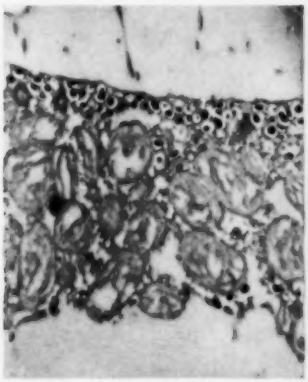
PLATE VII

FIGS. 21–22. Two of the sections (Nos. 1 and 3) from the diagram of Fig. 26, p. 66 to show plastid structure, cilia in TS (arrows), golgi and associated mitochondria, &c., ×20,000 (GS4/13 and 4/16).



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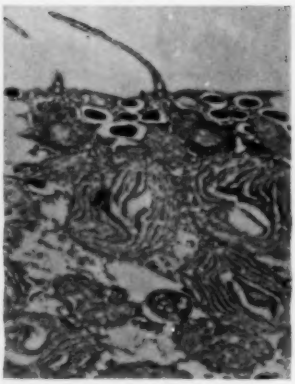

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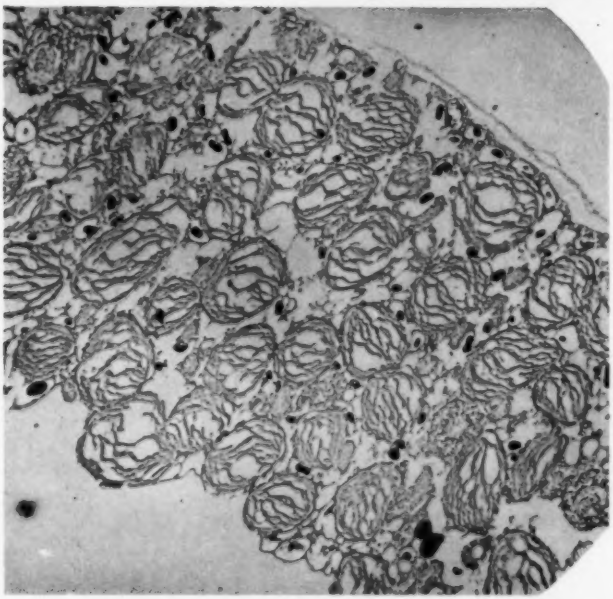

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PLATE VIII

FIG. 23. Section from surface to main vacuole of a motile spore (cilia visible externally) fixed in presence of 1 per cent. $CaCl_2$, showing conspicuous opaque contents in the peripheral vesicles, $\times c.$ 4,000 (G567/22).

FIG. 24. The surface of a similar spore fixed in presence of 0·5 per cent. $CaCl_2$, showing peripheral vesicles and two nuclei each with a ciliary base and part of attached cilium, $\times$8,000 (GS15/2).

FIG. 25. Section from surface to main vacuole of a settled spore (cf. Fig. 4) but fixed in presence of 1 per cent. $CaCl_2$. Vesicles with opaque contents present at all levels; the already compound cell wall lifted clear of the cytoplasm, $\times c.$ 5,000 (G468/10).

Geotropic Behaviour of Roots

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Received 28 February 1958

SUMMARY

Response of roots to a gravitational stimulus consisting of a sudden artificial change of orientation in the gravitational field can be resolved into a phase of very rapid curvature and reduced growth-rate which dies out rapidly and is followed by a phase of very slow response and normal growth-rate.

A somewhat similar two-phased response to sudden reorientation is found in certain plagio- and diageotropic organs. The mechanism is discussed.

Starch column chromatography has been used in an attempt to isolate growth factors which according to one postulate might be involved, and the significance of the findings is discussed.

This technique is shown to be convenient for effecting volatile active substances which are lost in paper chromatography.

PART I

So many reviews in journals (cf. Rawitscher, 1932; Brauner, 1954) and discussions in textbooks have been devoted to the question of the mechanisms both of geo-perception and response that we do not propose to cite literature except when necessary to the arguments advanced here.

Suffice it to state that the older classic hypothesis which involved statoliths or movable starch grains has been largely replaced by one in which auxin is held to accumulate on the lower sides of orthotropic organs displaced from their normal position. Both these views are discussed at some length in the excellent textbook or monograph by Rawitscher (1932). Neither of these opposed 'classic' hypotheses was adequate, though the statolith hypothesis was fairly satisfactory as an explanation of the first phase of geo perception but not of response, and the auxin hypothesis seemed plausible enough so far as response was concerned but failed to explain the mechanism of perception. Neither hypothesis could deal with plagio- or diageo-tropism (Bennet-Clark and Ball, 1951). The difficulties encountered in 'explaining' diageotropism were pointed out in that study and a hypothesis was put forward which, though covering certain aspects of the behaviour of rhizomes, was very incomplete and in many respects unproven. It did, however, lead to extensive studies of growth regulators by chromatographic techniques (Bennet-Clark, Tambiah, and Kefford, 1952) and to an attempted study of the influence of orientation on rates of growth and content of growth regulators some of which is reported here. The original intention was to compare rates of growth of roots and

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shoots in their normal positions in relation to gravity with the rates in the inverse position, since it had been found in the case of rhizomes of *Aegopodium* (Bennet-Clark and Ball, 1951) that the growth-rate of those pointing vertically upwards was less than that of those placed horizontal which again was less than that of those pointing downwards, a result in accord with the preliminary hypothesis advanced. The present study and the earlier work on *Aegopodium* have suggested some very general considerations on the mechanism of response of plants to gravity which will be developed in final discussion.

EXPERIMENTAL MATERIALS

Roots of mustard (*Sinapis alba*), bean (*Vicia faba* var. Windsor), and pea (*Pisum sativum var.* Meteor) were the main experimental material. They were grown in a constant temperature dark room under conditions which have already been described (Younis, 1950, 1954).

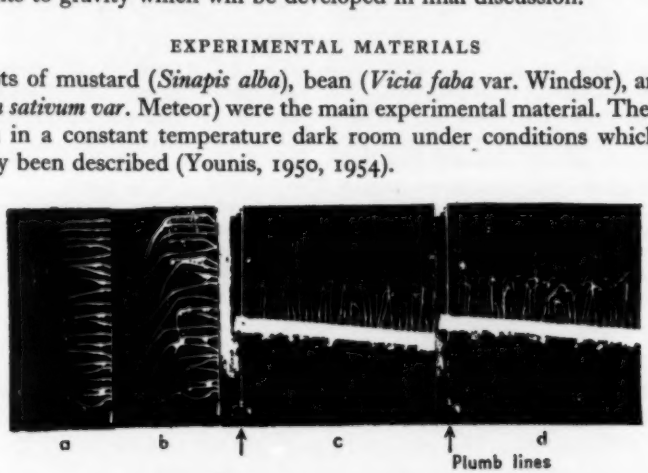


FIG. 1. Photographs on IR plates showing angles taken up by *Sinapis* roots: (*a*) 3 hours 50 min., (*b*) 22 hours 50 min. after placing at 90°, (*c*) 8 hours 50 min., (*d*) 23 hours 50 min. after placing at 180°.

A pad of flannel covered with cotton-wool and over this black cloth was supported on perspex and slid into a perspex box 20×20×5 cm. provided with a glass front or cover. The pad was kept uniformly moist by letting water drip through at about 300–500 ml./hour. Beans and peas were treated and mounted as described before (Younis, 1954). Mustard was germinated on pads of cloth about 200 seeds being sown on a wet pad which was then placed in a rectangular trough. Thirty-eight hours after sowing, seedlings with roots 12 mm. long were transferred to the pads in the experimental perspex troughs. Only about 10 per cent. of the seedlings sown proved to be suitable for transfer; seedlings with longer roots usually suffered damage to root hairs and this greatly affected later growth, even slight tearing of the root hairs caused a remarkable retardation of growth-rates.

An idea of the experimental arrangement can be seen in Fig. 1 which shows the general appearance of the experimental roots as shown on the infra-red sensitive plates which provided the photographic records of growth.

The chambers were arranged so that the angle made with the vertical could be adjusted according to experimental needs. Rates of growth and curvatures

were determined by projecting the infra-red plates on to a screen or wall and measuring the images with a protractor, thin steel ruler, and a map-measurer. Absolute dimensions were obtained by means of a scale photographed alongside the roots.

Certain special techniques are dealt with when individual experiments are described.

RESULTS

Effect of alteration of position on Sinapis roots. Seedlings were transferred at 38 hours old and 12 mm. long and after a further 24 hours were ready for experimental treatment. In each of the series of experiments to be described this timetable was followed: The batch of seedlings were photographed by red light on IR plates at zero hour. The chamber containing them was then turned through an angle and photographs on IR plates were taken at approximately 15-minute intervals during the phase of rapid movement (1 hour) and after that at 30-minute intervals for 2 hours and then at longer intervals.

In certain experiments some roots bent away from the moist pads and their subsequent growth was irregular and they were 'discarded'. This has caused irregular numbers to be available for measurement in the different experiments. The angles to the vertical and numbers of roots in each experiment are given below (the convention used is that the normal vertical-downwards angle is termed 0°, vertical upwards 180°): Expt. 13, 0° 21 roots; Expt. 14, 30° 24 roots; Expt. 15, 45° 11 roots; Expt. 16, 60° 22 roots; Expt. 17, 90° 13 roots; Expt. 18, 120° 32 roots; Expt. 19, 135° 13 roots; Expt. 20, 150° 18 roots; Expt. 21, 180° 17 roots.

Fig. 1 shows the appearances in the case of Expts. 17 and 21. The most cursory glance shows the remarkable facts that the roots of Expt. 17 placed horizontal do *not* curve into the vertical position and in Expt. 21 again they curve so that many of them are found to be growing roughly horizontal for a considerable time.

These reactions are most conveniently recorded by the method of Fig. 2, plotting angles taken up by the axes of the root tips as ordinates and times after stimulus as abscissae. The position before the stimulus was very close to 0°. The stimuli were given by turning the whole seedling chamber through 30°, 45°, 60°, 90°, 120°, 135°, 150°, and 180° respectively. The points plotted in Fig. 2 are the average of all the roots involved in each experimental treatment. It will be noted that in every case, there is a period of rapid bending lasting about 3 to 7 hours and after that very slow or little further bending. So that the final average angles adopted vary from 40° to 20° except for the series starting at 45° and 30°. This means of course that a large number of individual roots had not bent as much as this.

It is instructive to extract certain other data from these experiments. In Fig. 3 are plotted, for certain of these experiments, the mean rate of curvature in degrees/hour and the mean rate of elongation of the mid-line or axis of the root. These records make it clear that the gravitational or change of position

stimulus has the effect of causing a marked change of elongation rate and simultaneously a rapid positive geotropic curvature; the reduced rate of growth is not maintained for very long and as the growth-rate recovers so the ability of root to curve rapidly seems to disappear; the rapid rate of curvature peters out.

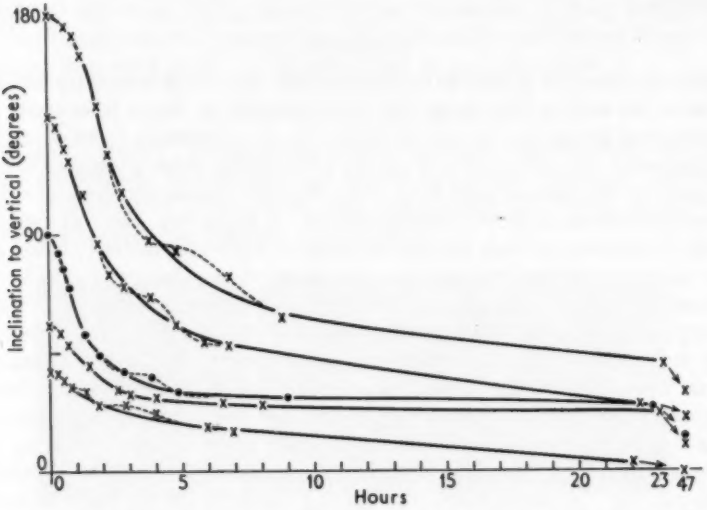


FIG. 2. Angles taken up by the apex of *Sinapis* roots at various times shown as abscissae after displacement from the normal vertical (0°) to a range of angles as shown by the zero hour readings (see text pp. 71 and 80). Note that the final reading was made about 24 hours after the penultimate one.

It can be very quickly seen from the records in Fig. 2 that the rate of bending at any given time is not proportional to the sine of the angle of inclination of the root tip at that particular time. If, for example, one considers the rate of response of root tips which are at an angle of 60° to the vertical, the rates of bending were 2·5°/hr. in the case of roots which had originally been at 180°, 7°/hr. and 33°/hr. for roots originally at 135° and 90° respectively.

Rather similar results were obtained when beans and peas were examined similarly. It was, however, noted that beans (*Vicia faba*) turned into the 180° position were much more sluggish in movement than mustard and frequently continued to grow straight upwards for 3 to 5 hours. In three separate experiments involving 9 to 5 roots each, results were as given in Fig. 4. In spite of the erratic behaviour the retardation consequent on the change to the inverse position is very clear. The behaviour was in all cases characteristic: there was a phase of rapid bending followed by a phase of relative inactivity. In the case of bean (*Vicia*) the roots frequently grew nearly horizontally for several days as though they were diageotropic.

This suggested a condition of fatigue, as the lack of curvature was noted although the gravitational stimulus (in the sense of non-vertical orientation) was being applied and any hormone-redistribution due to gravity would, one would expect, be operative. The fatigue phenomenon was re-examined using *Sinapis* seedlings as larger numbers and more satisfactorily based average

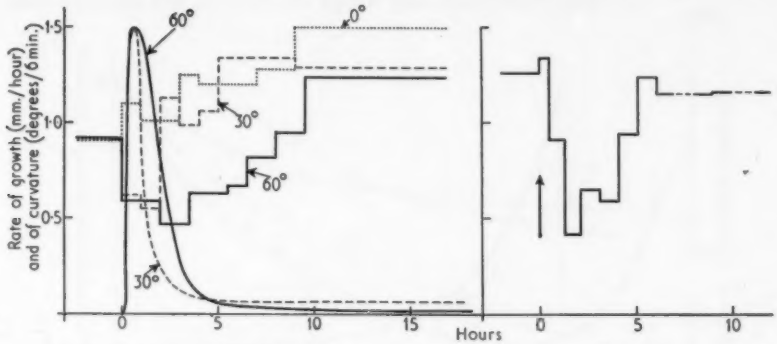


FIG. 3 FIG. 4

FIG. 3. Rates of extension, shown as histograms, of the mid-line of *Sinapis* roots subject to stimulation by displacements to 30° and 60° and control. Rates of curvature in degrees/6 min. are shown by the smooth curves.

FIG. 4. Rate of elongation of the mid-line of *Vicia faba* roots after displacement (at arrow) from 0° to 180°.

curvatures could more easily be obtained. In a typical experiment (Fig. 5), roots were stimulated by being turned through 135°; the rate of curvature slowed off typically from a maximum rate of 24°/hr. to about 3°/hr. at the 5th hour after the stimulus. The roots were then re-stimulated by being again turned through 90°; the rate of bending rapidly became 33°/hr. falling to about 5°/hr. after 5 hours and to an average of 1°/hr. in the next 15 hours.

Comparison of curvatures in moist air and other media. Since it is very usual to see either textbook pictures of demonstrations or even the demonstrations themselves showing seedling roots in process of curving into the vertical position, the results described seemed odd, and we examined the curvatures obtained when seedlings were grown in sand or soil in glass-sided boxes as devised by Sachs. Reasonably rapid curvature into the vertical position occurred. In fact, these phenomena, rapid curvature to a vertical position in soil and slow movement with failure to attain the vertical in moist air, were known to Sachs himself (1873) though the account given in his textbook and the much reproduced figure from it are misleading in that they do not tell the whole truth.

The importance of haptotropic or touch stimulation advanced by Sachs was examined in a series of experiments using roots of *Vicia faba* (var. Seville Longpod). Seed was germinated and seedlings were planted so that their roots grew (1) in moist air along the surfaces of moist cloth pads (as described

above, p. 70), (2) in soil, (3) in sterilized sand, (4) in fine smooth glass beads known as ballotini. The last three media were kept moist but not waterlogged. The vessels containing the seedlings were turned through 90° to provide the standard gravitational stimulus. The angles taken up by root apices at various times after the stimulus are shown in Fig. 6. It will be seen at once that

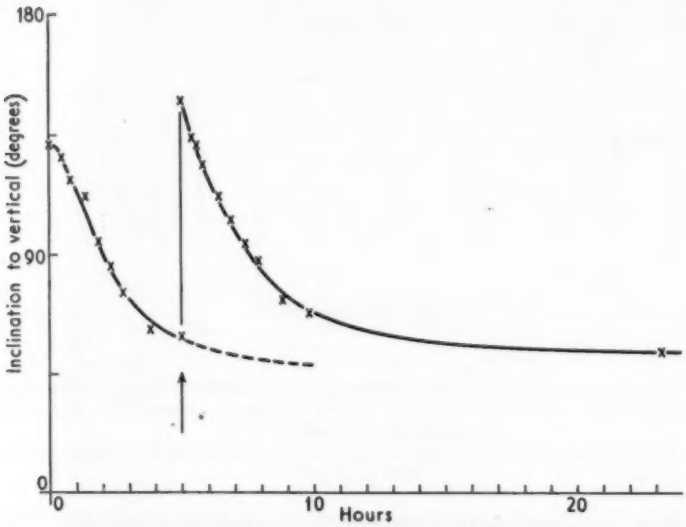


FIG. 5. Angles taken up by *Sinapis* roots following displacement from mean of 0° to a mean of 142°. At the arrow the partially recovered roots were restimulated by being turned through 90°.

series (1) and (4) grown in moist air and ballotini respectively curve much more slowly than series (2) and (3) in soil and sand. In all four series the rate of curvature remains relatively rapid during about 10 hours after which further curvature is very much slower.

This suggests strongly that in the case of series 2 and 3 the more rapid curvature may be due to the combined effects of geo- and hapto-tropism whereas in series 1 haptotropism is fairly certainly eliminated and it is also probably eliminated in series 2 as ballotini are extremely smooth and when moist must present a relatively frictionless surface to the growing root apices and one likely not to cause any mechanical damage to the roots. Sharp sand on the contrary may well cause superficial injury to a bending root.

Direct examination of touch or injury phenomena. Bean roots grown in moist air were subjected to a variety of touch stimulations. Roots grown vertically in moist air were touched with small pieces of emery paper on one side, and in some cases a small piece of emery was left stuck on the root for several hours. Rather irregular curvatures were observed but generally towards the touched side of the root.

The situation was more regular when roots were subjected to a gravitational stimulus by being turned into the horizontal position. Roots left horizontal in moist air were compared with similar roots to which a little emery had been applied on either the upper or lower side. This clearly showed enhancement of the reaction in those roots given a touch stimulus on the lower side and the contrary for upper side stimulated roots.

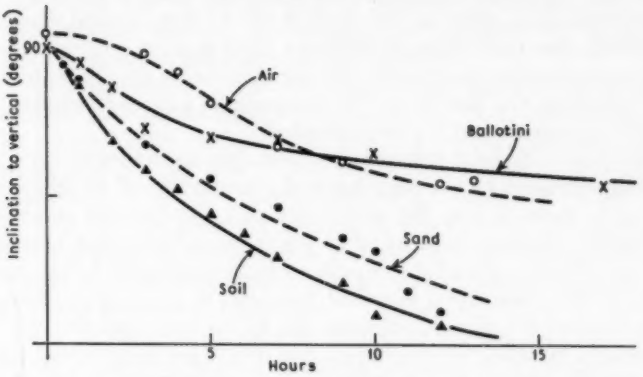


FIG. 6. Angles taken up by *Vicia faba* roots at various times (abscissae) after placing horizontal (90°) when grown in air, ballotini, sand, and soil.

Behaviour of other seedling roots. It should be emphasized that a considerable range of different behaviour patterns must exist in different species and varieties. Mustard, bean, and pea are (at least with the varieties used by us) unlikely to complete curvature into the vertical position in moist air within 10 hours and the very slow later response means that they may not become vertical for many days. On the other hand, we have observed very rapid response when roots of *Linum usitatissimum*, *Gilia capitata*, *Artemisia* sp. amongst others, were turned into a horizontal position whilst growing in moist air. There is therefore some quantitative difference in behaviour which requires explanation.

DISCUSSION

The noteworthy feature of root geotropism revealed in this study was in part discovered by and known to Sachs (1873), namely that in moist air certain roots began to effect a positive curvature but did not turn fully into the vertical position unless also in contact with soil particles.

The further point has been made clear in the present study that roots of mustard and bean turned through a range of angles do undergo positive geotropic curvature quickly for a limited period of time of between 3 to 10 hours after which very slow further curvature may occur. During this period of quick curvature, the rate of extension growth is markedly reduced. This strongly suggests that curvature is being caused by an inhibition which

is produced by the stimulus and which is effective on the lower side of the root. This inhibition of the growth of the lower side could be due to the production of an inhibitor there and the dying away of the tropic reaction during a period of about 10 hours due to the disappearance in metabolism of the 'dose of hormone' produced by the stimulus. A second stimulus in fact produces a second response, as shown in Fig. 5. In this case after the first stimulus the rate of curvature of the roots had decreased to a very low figure; the roots were then again turned through 90° so that the tips were at 160° again and the tips again began to execute a rapid positive geotropic response. This suggests that the dying out of the first response is not primarily due to a 'fatigue' phenomenon such as loss of nutrients, as a second response can be so quickly commenced after a new stimulus.

Of course it would be as satisfactory to postulate the destruction of a growth promoting substance by the stimulus as the production of an inhibitor. If a promoter be destroyed by the occurrence of a gravitational stimulus, one would have to postulate further that during the ensuring period, new production in metabolism replaced the growth promoters destroyed by the stimulus. Either postulate, destruction of growth promoter or production of inhibitor, has this feature: the stimulus consists not in the mere fact that the root is lying static in an abnormal orientation but that it has been turned suddenly on its side (or upside-down). This we suggest promotes quite abnormal chemical or physico-chemical changes, just as turning a cupboard full of chemical reagents on its side suddenly might well provoke chemical reactions.

It has become so usual to 'explain' tropisms in terms of redistribution of special substances (auxins) that one is inclined to forget that it might be as reasonable for the response to be caused by a release of material from a site in the cell where it is 'bound'. This site might be the vacuole and it is certainly true that various Leguminosae contain in the vacuoles of their roots concentrations of coumarin derivatives which supplied to the outside surface of the root completely stop growth.

The hypothesis of new production of growth substances or possibly their release from vacuoles has another feature more consistent with the experimental data than the earlier hypothesis of redistribution of auxin already present. This classical auxin-redistribution theory would lead one to expect a rate of response roughly proportional to $g \sin \alpha$ (α being the deflexion from the vertical), and as the value of α decreased as a result of the response so $g \sin \alpha$ and the rate of response would decrease. In fact the records of Fig. 3 and many other experiments show that there is very little proportionality between rate of response and $g \sin \alpha$. The rate of response is, however, rather closely connected with lapse of time since application of the sudden change of position.

The behaviour of roots in soil, sand, and other media is to be explained on the basis of combined effects of geo- and hapto-tropism. The first effect of the geotropic bending is to cause greater pressure of the sand particles on the lower side of the root and this side thus receives the greater haptotropic

stimulus. In the case of the experiment illustrated in Fig. 6, it will be noted that curvature is much quicker in sand and soil, than in air or ballotini in which media haptotropism is largely eliminated.

The data seem to suggest that the 'gravity hormone' has an effective lifetime of some 10 hours in bean or mustard, but the behaviour of some other roots as described (for example, *Gilia* and *Artemisia*) suggests that in these roots the persistence of the hormone is such that curvatures are complete within the effective lifetime. We imagine that this lifetime is dependent on rate of destruction in metabolism of the 'hormone'.

Regarding the mode of action of this gravity produced inhibitor, which we postulate, we have no information at present. Dr. N. Grobbelaar on a short visit here very kindly examined the effect of gravitational stimulation on respiration rate of bean root apices. Roots were grown in normal vertical orientation and others were turned through 90°. Apices were cut from the vertical roots and also from the others 2, 6, and 10 hours after placing horizontal. It was thought possible that at about the time which would correspond to the minimum growth-rate and maximum rate of curvature that there might be evidence of inhibition of respiration. This was not found.

Of course it could be that the inhibitory mechanism consisted of an uncoupling of phosphorylation or of energy transfers. It is perhaps even more likely that wound responses entirely swamped any effects associated with the geotropic stimuli given.

COMPARABLE PHENOMENA WITH OTHER ORGANS

Any hypothesis of geotropism, in order to be truly acceptable, must fulfil the condition of explaining the phenomena of plagiotropism and orthotropism of organs predominantly negatively or predominantly positively geotropic, or there must be acceptable reasons for the very diverse reactions of different plant organs.

It is therefore of some importance to consider whether this view of a hormone actually produced or released by the stimulus as opposed to a hormone redistributed under influence of the stimulus is likely to explain other phenomena.

Plagio- and diageo-tropic organs show characteristic responses to the stimulus of being suddenly deflected or moved out of their normal position of orientation. Some of these responses are schematically shown in Fig. 7.

Fig. 7 B illustrates the behaviour of runners of *Ranunculus* studied by Zimmermann (1924). The normal angle adopted is the upward slope (about 110°). If the runner is turned to the vertical up position, 180°, it bends in the sense shown by the bent arrow in the diagram. In other words, growth of the former lower side is inhibited and a so-called dorso-convex curvature occurs. This happens when the runner is deflected from its normal 110° to any angle between 110° and 360° and also to positions between 45° and 0° (or 360°). Deflexions to angles between 110° and 45°, however, result in dorso-concave curvatures as indicated by the bent arrow.

However, if the runners are turned from the normal to a wide range of angles and then held in the new angular positions for 12 hours so that they cannot bend and are then released, a quite different behaviour is found (shown in Fig. 7 c). Dorso-convex curvatures are found when the new angle is between 110° and 180° and between 0° and 250° as shown by the bent arrows; in all other positions dorso-concave bending occurs. In other words,

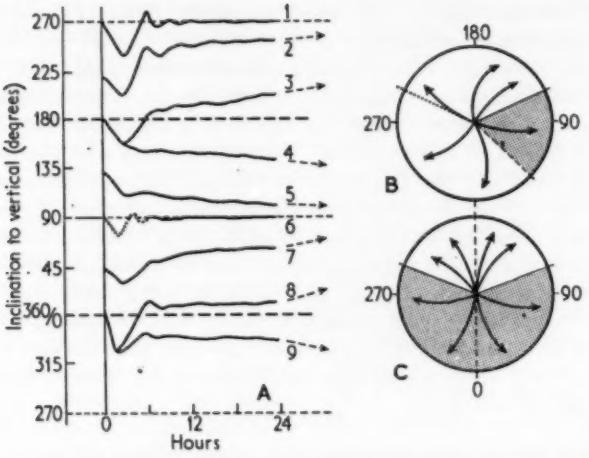


FIG. 7. A. Diagram to show the time course of angular displacement of tips of rhizomes of *Aegopodium* following displacement from their original horizontal position to the angles indicated at the zero hour abscissa (see text).

B. Type of curvature shown by runners of *Ranunculus* when displaced from their normal orientation (slope about 20° above horizontal) to a range of angles diagrammatically shown.

C. Similar responses by *Ranunculus* runners kept forcibly at the range of displaced angles for 12 hours and then released.

in this case the runner bends by the quickest available route towards one or other of the equilibrium positions of 110 or 250° (sloping upwards 20° above the horizontal). This suggests that something initiated by the change of position stimulus dies out after about 12 hours.

The situation is clarified somewhat by consideration of behaviour of a diageotropic rhizome such as *Aegopodium*, the behaviour of which is shown in Fig. 7 A (Bennet-Clark and Ball, 1951). The nine curves show the responses which follow turning the rhizomes from the normal horizontal position (90°) into 270° (i.e. turning through 180° into the horizontal position but upside down); 225°; 180° (vertical up); 135°; 45°; 0°.

It will be seen that there is a characteristic rapid bending towards what had been before the stimulus, the lower side of the rhizome; in other words a rapid dorso-convex curvature occurs here (as with runners) and this lasts about 3 hours and is followed during roughly the next 3 hours by an almost

equally rapid dorso-concave curvature. Rapid bending comes to an end by about the 7th hour and is followed by a very slow return of the rhizome towards one or other of the equilibrium positions 90° or 270°. The 180° and 0° (or 360°) positions are 'metastable': rhizomes turned into these positions show the dorso-convex curvatures followed by the reverse dorso-concave but these latter may be too weak in certain cases to overcome the tendency to return to the horizontal plane, so one finds in curves 4 and 9 behaviour contrasting with that shown in curves 3 and 8 where the dorso-concave reverse reactions are sufficiently strong to bring the rhizome back towards the opposite horizontal position. The gradual return of the rhizomes from the 7th–9th hour onwards is, as shown, very slow and may be towards either of the equilibrium positions, 90° and 270°, which in effect means that if displaced to the metastable positions 180° or 0°, the majority go towards 90° from 180° and towards 270° from 0° owing to the initial dorso-convex curvatures.

These initial rapid movements are essentially similar in runners of *Ranunculus* rhizomes and in diageotropic aerial branches such as those of *Asparagus* (Rawitscher, 1932) and do not necessarily follow the same course as the slower geotropic movement which restores the normal orientation.

The initial invariable dorso-convex bending implies a gravitational asymmetry, such that the effect of the stimulus (shock) produces inhibition of the lower side. A displacement of rhizomes of *Aegopodium* through 180° for only 10 min. induces this effect as shown by curve 6 (fig. 7) but the movement downwards only begins after the rhizome has been returned to its original position.

These facts are not easy to fit into a hypothesis which ascribes the geotropic movements as due to downward migration of auxin across the plant organ and a relatively direct effect of auxin on the extensibility of the growing cell (possibly through an effect on plasticity of the cell wall).

The behaviours of plagiotropic organs and roots would fit better a hypothesis that the geotropic stimulus produced an inhibitor *de novo* or caused its release from vacuole to cytoplasm. It would be necessary to suppose that a mechanism existed in the plant which caused the inhibitor to move downwards in the gravitational field so that it became more concentrated on the lower sides of horizontally placed or sloping plant structures and also that the precursor or 'vacuolar stock' is present in greater quantity on the lower sides of dia- or plagio-tropic organs.

These organs, plagio- and diageo-tropic runners, rhizomes, and roots, differ from aerial stems in that in Nature they are not subjected to sudden large angular displacements. At most, they are slowly deflected by obstacles. The response to such slow deflections may resemble the slow return to normal orientation which rhizomes and roots show some 10–12 hours after the sudden artificial stimuli which we have been considering. There is no specific evidence on this. The slow return to normal orientation clearly can be an upward (−) or downward (+) movement depending on the organ and direction of its displacement. The initial rapid response is always downward

(i.e. dorso-convex) but is followed quickly by a succession of back and forward movements which would be called autotropic and for which there are mechanical analogues in servo-mechanisms but for which we have no chemical or physiological picture yet. There are indications of a similar time course of the response of orthogeotropic roots in the data of Fig. 2. The experimental points have been fitted by eye to the smooth curves and dotted curves which latter are in fact the closer fit. Examination of individual roots usually reveals to and fro bending movements.

These observations appear to support and confirm the point of view that has been expressed regarding root geotropic mechanisms by Audus and Brownbridge (1957).

We have no completely satisfactory hypothesis of a mechanism to bring about displacement of material across an organ in the gravitational field: this, however, must occur as many rhizomes are biochemically but not anatomically dorsiventral. Nor can we yet suggest a mechanism by which a sudden deflexion through a large angle can release or produce inhibitors or accelerators though we suggest that the mixing up of cell organelles by displacement of heavy starch grains may be part of the mechanism.

PART II

R. ESNAULT

The previous section has suggested that a specific hormone might be formed by sudden deflexion of roots through a large angle. Instead of one substance which it was suggested disappears in metabolism, the stimulus might produce a complex pattern of substances which could influence growth-rates. This has indeed been suggested as more probable by Dr. R. Brown (1958).

An attempt has therefore been made to ascertain whether roots left growing normally in the vertical-down position contain a different complex of hormones from those which have been suddenly deflected through 90°. The analysis of growth factor contents has been attempted by chromatography. In this connexion one notes some discrepancies in the claims of various workers. We have attempted to examine this question again by a chromatographic technique which we believe has not previously been used with extracts of plant material.

As IAA has been regarded as of major importance, we have paid special attention to the identification or recognition of factors which might possibly be confused with IAA on the basis of chromatography.

METHODS

Three major problems have to be faced. It is necessary to prevent the possible enzymatic release or production of auxin from precursors or from substances such as 3-indolylacetylaspartic acid which may be regarded as detoxication products. The only really satisfactory procedure is a short period

of boiling of the tissue which will destroy enzymes. This treatment may of course destroy certain hormones but free IAA is certainly not destroyed.

Since estimates of the IAA content of tissues are commonly in the region of a few μg. per kg. tissue, relatively large amounts of tissue have to be extracted and the extract evaporated or suitably concentrated to provide the starting spot of a paper chromatogram. One may want to obtain from 100 g. tissue one single starting spot of a chromatogram and this clearly demands separation of the auxin from the great mass of other solutes.

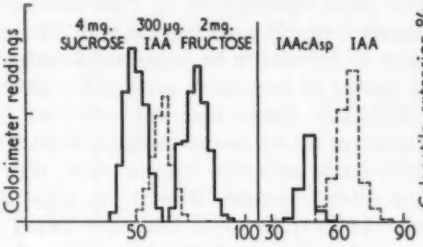


FIG. 8

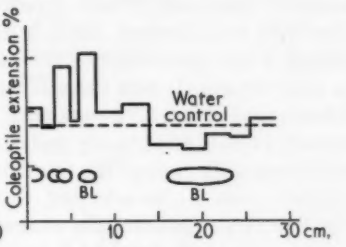


FIG. 9

FIG. 8. Histograms showing starch column separations of IAA/300 μg. from sucrose 4 mg., fructose 2 mg., and from indolylacetylaspartic acid. Ordinates are arbitrary colorimeter readings; fraction numbers are shown as abscissae.

FIG. 9. Paper chromatogram of extract from 10 g. *Vicia* root apices; acid fraction developed in *iso*propanol-ammonia; oat coleoptile extension test. UV fluorescent zones are shown (Expt. 11 *d*).

The method developed here was extraction by alcohol, evaporation and transfer to water, extraction from the aqueous solution by ethyl acetate, followed by extraction from the ethyl acetate by water at pH 8 ($NaHCO_3$), acidification and further transfer to either ether or ethylacetate. The final solution contains the auxin but also contains various coloured substances and a considerable weight of other largely unidentified material which interferes with the running of the chromatogram, in certain cases very seriously.

Further initial purification is therefore necessary and we decided to use the starch column technique of Lathe and Ruthven (1956) which effects a separation of substances that appears to be based largely on molecular size or at least on penetrability into starch grains.

Starch columns were prepared as described by Lathe and Ruthven using BDH potato starch run into tubing of 18 mm. internal diameter. The standard filling found convenient was about 90 g. of 'bottle' potato starch corresponding to 70 g. dry starch. Fig. 8 gives an example of a trial separation of solutes on such a column. The relative quantities of sucrose, fructose, and IAA in the series of fractions obtained by a fraction collector are given. This shows that the IAA is likely to be readily separated from a considerable number of substances of different molecular size by this technique.

The IAA containing fractions can be identified by colorimetric assay (as in Fig. 8) or by bio-assay and considerably more unreliably by the R value or total eluate volume. These fractions separated on basis of molecular size or permeability to starch grains can then be still further fractionated on a partition coefficient basis by paper chromatography.

Starch column separations of IAA and IAc-Aspartic acid. It has been shown in this Department that IAA is rapidly converted in many tissues into 3-indolylacetylaspartic acid (IAcAsp) as had previously been found by Andreae and Good (1955). Dr. Wheeler working here has shown also that the naturally occurring growth promoter zone described as 'α' (Bennet-Clark, Tambiah, and Kefford, 1952) is almost certainly not identical with IAcAsp though it has approximately the same Rf (results to be published *in extenso* in near future). It was therefore a matter of interest to carry out a starch column separation. This was done as before: the column was loaded with a mixture of IAA and IAcAsp and contents of the successive fractions estimated colorimetrically using the perchloric-ferric chloride reagent with which IAcAsp gives a characteristic violet colour reaction. Results are given in Fig. 8, and, as expected, the somewhat larger molecule of IAcAsp is found in fractions just before the IAA fractions. In other words the Rf is larger than that of IAA whereas with partition chromatography it is smaller.

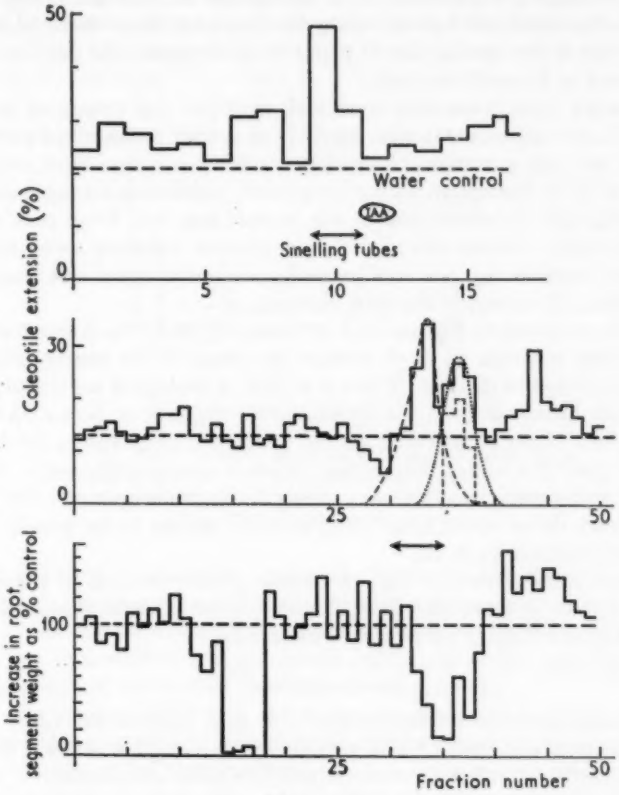
Simple partition chromatography of root tip material. Results of straightforward extraction and paper chromatography are given in Fig. 9. Bean (*Vicia faba*) root tips (10 g.) were boiled in 20 ml. methanol for a few minutes and were macerated in a MSE macerator. The methanol was evaporated off from the clear extract and the watery solution left was extracted three times with ethyl acetate. The ester layer was separated, extracted with 10 per cent. $NaHCO_3$ and the bicarbonate solution acidified with phosphoric acid to pH3 and re-extracted with ethyl acetate. This final ester solution was evaporated and completely transferred to the starting spot of a chromatogram, which was developed by *iso*propanol–ammonia–water (100:10:10) in the usual way.

Bio-assay of regions showing defined fluorescence colour when illuminated by UV radiation was carried out, using *Avena* coleoptile sections. These regions are shown diagrammatically towards the top of Fig. 9; the position of an IAA marker spot as shown by the perchloric acid spray was between 6 and 8 cm. from the starting line.

It will be noted that material in 'the IAA position' is present. A fluorescent spot of smaller Rf is also present and shows smaller but significant growth promoting activity. Comparison of this result with chromatograms obtained under similar conditions by Kefford (1955) and others suggests that this spot of smaller Rf than IAA might be identical with his 'α' and that IAA and the inhibitor zone 'β' are also present.

Starch column separation. In a typical experiment, 100 g. of bean-root material essentially similar to that just described was subjected to the same technique of killing and extraction, up till the final extraction by ethyl acetate. This ethyl acetate was evaporated and the substances present (namely acidic

ester-soluble materials) were transferred in a small volume of water to the top of a 70 g. column of starch in an 18 mm. tube. This was eluted with water at a rate of about 3 to 4 ml./hr., and 5 ml. fractions were serially collected by an automatic collector.



FIGS. 10–12.

FIG. 10. Starch column separation (Expt. 11 *b*) of extract similar to that used for Fig. 9. Coleoptile extension test.

FIG. 11. Starch column separation of similar extract (Expt. 11 *d* I) to that of Fig. 10 using 100 g. root apices to which 100 μg. IAA had been added. Ordinates show coleoptile extensions (continuous line histogram) or colorimeter readings showing IAA content (dotted histogram). See text p. 84.

FIG. 12. Bio-assay using pea root extension (Expt. 11 *d* III) of fractions from a starch column separation similar to those of Figs. 9 to 11.

The results of the bio-assay carried out on 1 ml. samples from each fraction are given in Fig. 10. The position of the fraction in which IAA would be expected to appear is also indicated. Growth activity shown by bio-assay is

therefore close to, but definitely not identical with the expected position of IAA. Incidentally, this growth activity is, in the case of this plant material, associated with some substance having a faint characteristic smell. As there is clearly doubt regarding the identity or non-identity of the growth substance from the 'smelling tubes' with IAA, the groups of fractions having activity were further analysed by extracting the material from them with ether, transferring to the starting line of paper chromatograms and developing with *iso*propanol or butanol ammonia.

As results were somewhat equivocal, they are not presented here and several further experiments were carried out exactly as described except that the root tips (100 g. samples) immediately after maceration were mixed with 100 μg. of IAA. Extraction with ethyl acetate, starch column separation, and chromatography then followed in the normal manner. From each fraction from the starch column one aliquot was used for bio-assay using coleoptile extension, another for pea root extension in many cases, and another for colorimetric estimation of the IAA content.

Results are shown in Fig. 11. IAA contents, shown by the dotted histogram, indicate that fractions 34 to 38 contain the whole of the 100 μg. which was artificially added to the brei. There is a peak of biological activity shown by the growth histogram (and the dotted curve) which is evidently due to this added IAA. There is, however, a clear peak of activity shown by fractions 30 to 35 (and the broken line curve) which is considerably larger than the peak due to the artificial addition of 100 μg. IAA; this is evidently due to some other growth factor which is *not* IAA; the same applies to the growth activity shown by fractions 40 to 50.

Pea root assay shown in Fig. 12 reveals activity in each of these zones. Fractions 30 to 35 cause inhibition, fractions 40 to 50 cause acceleration, and fractions 13 to 18 cause very marked inhibition.

DISCUSSION

Simple partition chromatography of root apex material can easily suggest if one is uncritical that IAA is present in considerable amount and that possibly another or other accelerators and inhibitors are present.

The starch column separation shows, however, that the 'apparent IAA' is almost certainly not IAA. In Expt. 11 *d* 1 (Fig. 11) fractions 31 to 35 had a greater total activity than would have been given by IAA in the roots at a concentration of 1 mg./kg. This is not due to IAA as direct colorimetry showed that the artificially added IAA was in fractions 36 to 38. These two groups of fractions were then bulked and the contents of each were separately subjected to paper chromatography using *iso*propanol-ammonia as developer. Fractions 36 to 38 showed as one expected a very strongly marked peak of activity with Rf about 0·3 due to IAA. Fractions 31 to 35 which showed greater activity than fractions 36 to 38 yielded paper chromatograms from which little or no growth substance activity could be eluted (see Fig. 13). The conclusions to be drawn are that the active material of fractions 31 to 35

is either volatile and thus lost on drying the paper; or unstable, which seems unlikely in view of its stability in the original fractions. The method employed here, using starch column chromatography, gives one a very convenient technique for dealing with volatile growth substances.

This work began in an attempt to carry the analysis of geo-perception further. Roots were grown in normal orientation and portions of the crop

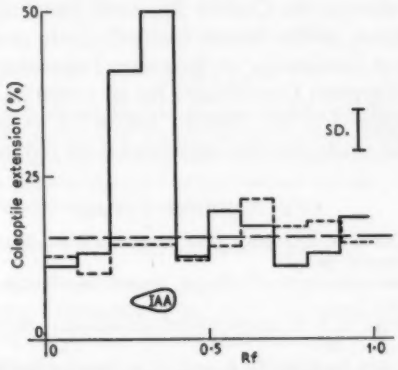


FIG. 13. Paper chromatograms using *iso*propanol-ammonia as developing solvent (coleoptile assay) of Fractions 31–35 of Fig. 11 shown by dotted histogram; and of Fractions 36–38 of Fig. 11 as continuous histogram.

(*Vicia faba*) were stimulated by turning the boxes in which they were growing through 90°. Samples were collected at zero hour and after 5, 12, and 18 hours from both stimulated and control roots. Chromatography and starch column separations as described did not reveal differences in the apparent growth substance content as between the different treatments.

One may conclude then that of the two alternatives discussed in Part I of this paper, that release of material from vacuole to cytoplasm is more probable as a mechanism rather than production *de novo* of a growth substance. Two of the separated fractions (Fig. 12) appear to have marked activity as root growth inhibitors but we have no means, as yet, of deciding if either of them contains substances which exercise functions of growth control effecting geotropic curvatures.

Until some such function has been revealed, there seems rather little point in proceeding with the very laborious and expensive process of separating from the crude 'active fractions' the major active constituents with a view to their ultimate identification and characterization.

Summarizing, one can state that IAA is present either in very low concentration or is absent, but that other material with Rf near that of IAA is present and some has activity of the same order that would be shown by IAA at about 1 p.p.m.; one of the separated fractions is apparently a volatile

'growth substance'. There is no evidence relating control of geotropic curvatures with any of the 'growth substance' fractions separated.

Correlation of the results reported here with those obtained by Audus and Gunning (1958) will require comparison of individual eluted fractions by the different techniques and is not possible at present.

ACKNOWLEDGEMENTS

Our thanks are due to the Central Research Fund of the University of London for apparatus; to the Centre nationale de la recherche scientifique and the University of London for an Exchange Fellowship tenable by one of us (R. E.) and the Egyptian Government for an award (A. F. Y.).

We are much indebted to Mr. Edward Adams for technical assistance and to Mr. J. Moon who made the first experiments on root haptotropism.

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Shikimic Acid in the Leaves of *Eucalyptus sieberiana* F. Muell.

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Received 17 June 1958

SUMMARY

Shikimic acid was determined in leaves, at different stages of growth, from 1- and 4-year-old specimens of *Eucalyptus sieberiana* F. Muell. A linear relationship was found between the shikimic acid concentration (mg./g.) and the weight of the leaf up to the point where the leaf had reached full size. As the leaves became older the shikimic acid content decreased.

INTRODUCTION

SHIKIMIC acid has been found in the leaves of a wide range of plants (Hattori *et al.*, 1954; Hasegawa *et al.*, 1957; Catravas and Kirby, 1948; Anet *et al.*, 1957). Davis (1955) has shown that shikimic acid is an intermediate in the path from carbohydrate to aromatic substances. Labelled lignin (Brown and Neish, 1955; Eberhardt and Schubert, 1956) and labelled quercetin (Underhill *et al.*, 1957) have been synthesized when labelled shikimic acid was fed to different plants. It could be expected that the greatest amount of shikimic acid would be found in the spring and summer growth of leaves at the time when the greatest amount of xylem is formed.

EXPERIMENTAL

Considerable quantities of shikimic acid were detected in the alcohol extracts of young leaves of *Eucalyptus sieberiana* F. Muell. by means of paper chromatography. Its identity was confirmed by comparison with authentic material using different developing solvents; (Hattori *et al.*, 1954; Hathway, 1956) and the specific spray reagent of sodium periodate-aniline (Yoshida and Hasegawa, 1957).

During the spring and early summer of 1957, large twigs were taken from 1- and 4-year-old specimens of *E. sieberiana* at about 8.00 a.m. Care was taken to choose twigs with leaves free from visible insect or other damage, and with the minimum number of stunted leaves. Within 0·75–1·5 hr of collection, single leaves were taken, extracted completely with ethanol, and the extracts analysed by a method essentially the same as that of Yoshida and Hasegawa (1957). In all, 120 leaves on 15 twigs collected from 5 trees were analysed and the results of duplicate analyses were, in most cases, within 2 per cent. When known amounts of shikimic acid were added to leaf extracts the expected increases were observed on analysis. Attempts were made to isolate shikimic acid with the aid of the ion exchange resin 'Amberlite 1 RA 410' in order to

confirm the analytical method. The crude shikimic acid was contaminated with a colourless substance (m.p. > 280° C.) but solvent purification produced increasing quantities of this substance. An approximate estimation of the polyphenol content was made by determining the absorption between 275–285 mμ on a Unicam S.P. 500 spectrophotometer.

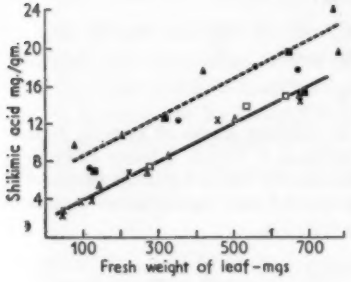

FIG. 1

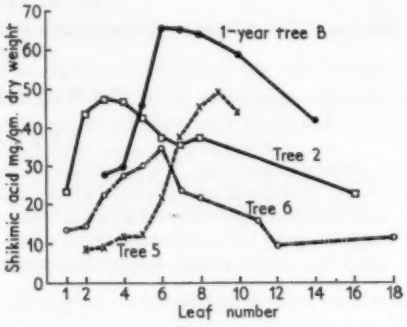

FIG. 2

FIG. 1. Lower line, three separate November collections of enlarging leaves from two 4-year-old trees (△, □, X). Upper line, three separate November collections from two 1-year-old trees (▲, ■, ●).

FIG. 2. Shikimic acid in leaves from representative twigs of *E. sieberiana* collected in November, tree 6 having chlorophyll-deficiency.

RESULTS AND DISCUSSION

When the leaves were enlarging, there was a linear increase in the amount of shikimic acid with the increase in weight of the leaves (Fig. 1). The maximum amount of shikimic acid was present in the leaf which had just reached full size. The shikimic acid content of fully developed leaves decreased with the age of the leaf (see representative examples, Fig. 2), and when they were more than 12 months old it decreased steadily from 18·9 to 10·9 mg./g. dry weight. The amount of shikimic acid in stunted leaves was higher than normal, for example leaf 8 of tree 2 (Fig. 2).

The change from the 'juvenile' to the 'mature' leaf form during growth of the tree could be seen in the width and weight of the leaves from the 1-year-old *E. sieberiana* trees which were 25–50 per cent. greater than those of the same length from the 4-year-old trees. The ratio of shikimic acid to leaf weight was also higher (Figs. 1 and 2).

The results show that the youngest full-sized leaves produced during the spring and summer contain the highest amount of shikimic acid. Thus, if shikimic acid formed in the leaves is translocated to the cambium to act as a precursor for lignin (and other materials), it is formed in greatest amount at the time when its consumption would be highest. The shikimic acid could give rise to polyphenols in the leaves but no relationship was found between the amounts of it and these materials.

The author wishes to thank Dr. Masao Hasegawa for his co-operation.

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Bicarbonate Ions as a Source of Carbon Dioxide for Photosynthesis

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Received 28 April 1958

FROM the observation that the rate of photosynthesis of some aquatic plants increased if the concentration of bicarbonate ions in a solution was increased while the concentration of free carbon dioxide was left constant, many authors have drawn the same conclusion as have Steemann Nielsen and Jensen (1958); namely that some 'aquatic species are able to utilize bicarbonate ions as well as free CO_2'.

Rabinowitch (1951) suggests that the lower rate with the lower concentration of bicarbonate ions may be due to what he calls 'exhaustion' or 'depletion' of carbon dioxide, but he does not consider the problem further. He does consider the problem of the maximum rate of production of CO_2 in a bicarbonate buffer relative to the maximum rate of consumption of carbon dioxide by a suspension of *Chlorella* cells.

The purpose of this paper is not to discuss all the evidence which has been advanced in support of the view that bicarbonate ions can be utilized directly in the photosynthetic process but to show that in some systems the observation recorded in the opening paragraph is compatible with CO_2 being the reactant and that the effect of the bicarbonate ion is to increase the concentration of the CO_2 in the interior of the system where it is being consumed.

Let us consider the rate of photosynthesis of a large plane sheet of thickness X cm. on the assumption that only CO_2 molecules are consumed. For simplicity the production of respiratory carbon dioxide will be neglected. If the sheet is exposed on both sides to a solution, large enough for the concentration to remain essentially constant with time, and stirred so rapidly that the concentration of carbon dioxide molecules and bicarbonate ions at the surface will be the same as in the bulk of the solution, c_0 and b_0 respectively, then in the steady state the following equation will hold for c, the concentration of CO_2 molecules in a plane parallel to and distant x from, the surface

$$K.d^2c/dx^2+A-Bc-k_3c = 0,$$

where K is the coefficient of diffusion of CO_2 molecules, A the rate of production of CO_2 per cm^3 from H_2CO_3 and HCO_3 ions, Bc the rate of conversion of CO_2 to H_2CO_3 and HCO_3 ions, and k_3c the rate of consumption of CO_2 molecules per cm^3 in photosynthesis. The solution of this equation is difficult if k_3, A, or B are not constant. There is evidence that for low concentrations, k_3 rises very little as the concentration of CO_2 falls. If the gradients of OH and

HCO_3 ions in the tissue are negligible then A and B can be treated as constants and $A = Bc_0$. It is sufficient here to note that if they are not negligible then the difference between the photosynthesis in a solution containing bicarbonate and one without will be less.

For a solution with no bicarbonate ions, A and B are zero and the equation for c for $X > x > 0$ is

$$c = c_0[\sinh \mu_1(X-x)+\sinh \mu_1 x]/\sinh \mu_1 X, \tag{1}$$

where $\mu_1^2 = k_3/K$.

The photosynthesis per cm^3, R_1, is

$$R_1 = k_3 c_0\left[\frac{2}{\mu_1 X}.\frac{\cosh \mu_1 X-1}{\sinh \mu_1 X}\right]. \tag{2}$$

If bicarbonate ions are present so that A and B are not zero

$$c = \frac{k_3 c_0}{k_3+B}\left[\cosh \mu_2 x-\sinh \mu_2 x.\tanh\frac{\mu_2 X}{2}-1\right]+c_0, \tag{3}$$

where $\mu^2 = (k_3+B)/K$.

The photosynthesis per cm^3, R_2, is

$$R_2 = k_3 c_0\left[\frac{2k_3}{(k_3+B)\mu_2 X}.\frac{\cosh \mu_2 X-1}{\sinh \mu_2 X}+\frac{B}{k_3+B}\right]. \tag{4}$$

In so far as the bracketed term in equation (2) falls below unity the rate of photosynthesis can be said to be limited by diffusive supply of CO_2; this is probably what Rabinowitch would call exhaustion or depletion of CO_2. The term approaches unity as $\mu_1 X$ approaches zero, that is as the thickness of the material approaches zero and as k_3, the coefficient for photosynthetic consumption, becomes negligible compared with K, the coefficient of diffusion. If the plant material has no intercellular spaces then K may have a value close to that in water, say 10^{-5} cm^2. $sec.^{-1}$ Data from which k_3 can be estimated are not common. For correct values the system should be such that the rate of photosynthesis is not limited by diffusion and the rate per unit volume is required at a concentration of carbon dioxide in the region where rate approaches proportionality to concentration. Data for *Chlorella* which satisfy these conditions indicate values for k_3 as high as 100 $sec.^{-1}$ The results obtained by van den Honert (1930) with a filamentous alga, *Hormidium*, give a value about one-tenth as big if we assume a fresh weight/dry weight ratio of ten. van den Honert is of the opinion that the rate is limited by diffusion and hence 10 $sec.^{-1}$ is an underestimate of the value of k_3. With k_3 as low as 10 the value of μ_1 is 1,000 $cm.^{-1}$ If the value of X is as small as 0·01 cm., or five cells of diameter 20 microns, the bracketed term is as small as 0·2. If X is reduced to a half this term, it still is less than 0·4, and less than 0·7 when X is reduced to a quarter. If the plant material contained spaces filled with air, where K has a value 10^4 times that in water, the bracketed term would be nearer unity.

When we turn to the effect of the presence of bicarbonate ions on the rate,

that is the relative dimensions of R_2 and R_1, then since the bracketed term in equation (4) cannot exceed unity, one of the conditions for the ratio R_2/R_1 being bigger than unity is for the bracketed term in equation (2) to be appreciably less than unity. The other factor is the relative dimensions of B and k_3. As we have seen $B.c$ is the rate of conversion of CO_2 to H_2CO_3 and HCO_3 ions. That part of B relating to conversion to HCO_3 ions depends upon the concentration of OH ions, that is upon the ratio of HCO_3 ions to CO_2 plus H_2CO_3 molecules. For a solution of $KHCO_3$ this ratio is about 100. Under these conditions the component of B for conversion to HCO_3 ions is about 5×10^{-3} sec.$^{-1}$ and that for conversion to H_2CO_3, which is independent of pH, is 25×10^{-3} sec.$^{-1}$ Hence B has a value of 3×10^{-2} sec.$^{-1}$ which is small compared with k_3 even if this is as small as 10 sec.$^{-1}$ The first term in the bracket of equation (4) will be essentially the same as the bracketed term in equation (2) and the last term in equation (4) will be 3×10^{-3}. For the first term to be as small as 3×10^{-3}, $\mu_2 X$, which is essentially the same as $\mu_1 X$, must be as great as $6{\cdot}7\times10^{2}$. With $\mu_1 = 10^3$ cm.$^{-1}$ X would have to be 0·67 cm. Under such conditions R_2 would be twice as great as R_1. With B small relative to k_3 any increase in the latter will result in a decrease much greater in the term $B/(k_3+B)$ of equation (4) than in the bracketed term of equation (2), that is the ratio of R_2 to R_1 would be reduced.

In comparing R_2 with R_1 we have used the value of B from the data of Brinkman, Margaria, and Roughton (1933) for the uncatalysed interconversion of CO_2, H_2CO_3 and HCO_3 ions. If, as in some photosynthetic material, carbonic anhydrase is present, then the coefficient B would be greater. If it were ten times as great, that is 0·3 sec.$^{-1}$, the last term in the bracket of equation (4) would be 3×10^{-2} if k_3 was still 10 sec.$^{-1}$ and for R_2 to be twice as big as R_1 X need be only 0·067 cm. If k_3 was as great as 100 sec.$^{-1}$ then this last term would be 3×10^{-3} and for R_2 to be twice R_1 the thickness X need be only $0{\cdot}67/3{\cdot}16$, or 0·21 cm., since μ_1 is increased from 10^3 to $3{\cdot}16\times10^3$ cm.$^{-1}$

Without considering systems of different shape the above suffices to show how factors such as the distance of photosynthetic centres from the surface of the system, the activity of the material per unit volume, and the concentration of carbonic anhydrase, which governs the rate of generation of CO_2, contribute to the determination of the effect of bicarbonate ions on photosynthesis via their effect on the concentration of CO_2 at the place where this is consumed.

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The Role of Boron in Plant Growth

II. THE EFFECT ON GROWTH OF THE RADICLE

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Received 31 July 1958

SUMMARY

Estimates of cell number, cell volume, respiratory rate, nitrogen, sugar, and nucleic acid content were made on 1 mm. sections of the radicles of field bean at frequent intervals during the first 96 hours of growth in nutrient solutions with and without boron.

The primary effects of deficiency were cessation of cell division and enlargement of the apical cells. The increased volume of the apical cells may have been due to either a longer period being available for development as the rate of mitosis decreased, or to an unusually rapid rate of cell extension. The results indicated that cell division did not cease for lack of available sugar nor as a result of failure to synthesize protein or nucleic acids. It is suggested that in the absence of boron division ceases because abnormalities in the formation of the cell wall prevent the cell from becoming organized for mitosis. In particular the hypothesis that boron is concerned with the formation of 'pectin' from uridinediphosphate-D-glucose is examined in the light of published evidence.

INTRODUCTION

EARLIER experiments (Whittington, 1957) emphasized the important relationship that exists between the supply of boron and cell division. There is, however, a lack of knowledge about the changes that occur in the meristem while deficiency is developing. The results of analyses of organs in which deficiency symptoms have long been apparent do not readily allow the separation of primary from secondary effects. A reduction in the time intervals between analyses during the onset of deficiency reduces, although it does not eliminate, this source of difficulty. The results now presented deal with the changes that occur in the radicle of field bean during the first 4 days of growth in a nutrient solution lacking boron.

METHODS AND MATERIALS

Seeds of spring field beans (supplied by Dunns Farm Seeds Ltd., Salisbury) were germinated on moist cotton-wool in metal petri-dishes at a temperature of 25° C. The seedlings were transferred to a bitumen-painted germination tank of 25 litres capacity when their radicles were about 2 cm. long. They were supported by 'Perspex' sheets in which holes had been drilled to allow the radicle to enter the nutrient solution beneath. The tank was kept at 27° C. in a dark room. Certain of the results were obtained from plants grown in one-pound jam jars, with glass covers drilled to hold five seedlings, and

maintained at 25° C. The nutrient solution was that previously used, with boron supplied where required at 1·0 p.p.m. Harvests were taken at 12-hour intervals after the roots were placed in the nutrient and continued for 96 hours. A razor blade screwed to a 'Perspex' body was used to cut a series of ten 1 mm. sections from each root. Throughout the experiments the apex of the root is referred to as section 1 and the remainder are numbered successively. The results for section 10 have been omitted since they are similar to those of section 9.

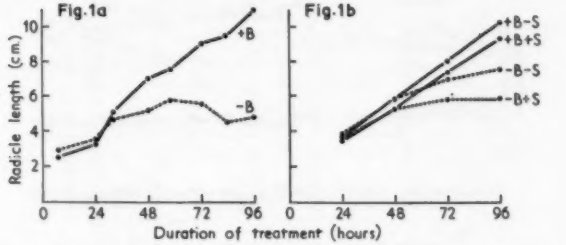
Dry weights were obtained using 30–60 roots for each harvest and total nitrogen was later determined on this material using the micro-Kjeldahl technique. The mean diameter of the sections from the ten roots were determined using a travelling microscope. Cell volume was estimated from this measurement, the length of the segment (1 mm.), and cell number per section. Respiration rate was determined from 30 roots in a Warburg apparatus, and this material was then fixed in acetic-alcohol and stored for subsequent determination of cell number. Before examination the material was washed very thoroughly to remove the fixative and was then macerated in 5 per cent. chromic acid at 45° C. until the sections could be broken down easily by repeated ejection through a fine pipette. Eight to fourteen separate determinations were made on each group using a haemocytometer. Estimation of total sugar was made on the acid-hydrolysed extract from ten roots using Somogyi's (1952) method. I am indebted to Dr. J. K. Heyes of the A.R.C. Unit of Plant Cell Physiology for the estimation of ribose (RNA) and deoxyribose (DNA) nucleic acids by U/V absorption and the method of Schmidt and Thannhauser (1945) respectively.

RESULTS

(*a*) *Effect on radicle growth.* The mean radicle length from 10 roots at 8 harvests showed that there was no further increase in length of deficient roots after about 30 hours of treatment (Fig. 1*a*). The precise time of onset of deficiency, however, varied between experiments. There were no differences in the fresh and dry weights per radicle between the two treatments up to 96 hours but at a later harvest the total root weight in the minus-boron plants was found to be lower than in the plus-boron series because the latter had considerably more functional meristems. Development of lateral roots took place normally in plus-boron roots at a distance greater than 1 cm. from the apex of the radicle. In deficient roots abortive lateral initials were formed within the apical centimetre of tissue particularly in sections 7–10 and also within the apex. This means that the comparisons between the two types of radicle were made difficult as soon as the minus-boron root ceased to grow. The comparison of the first centimetre of the deficient root with one centimetre of the plus root from the area forming laterals would be no improvement since, after the cessation of mitosis, the cells of the meristem of the deficient root do not become as large as the elongated cells of the normal root. The most appropriate comparison is that between the apical regions

of roots of the two series, but the results need to be interpreted with caution.

(*b*) *Effect of the presence of sucrose in the external medium.* In view of the work of Gauch and Dugger (1953) the effect of supplying sucrose in the culture medium was examined (Fig. 1*b*). Sucrose at 2 per cent. concentration



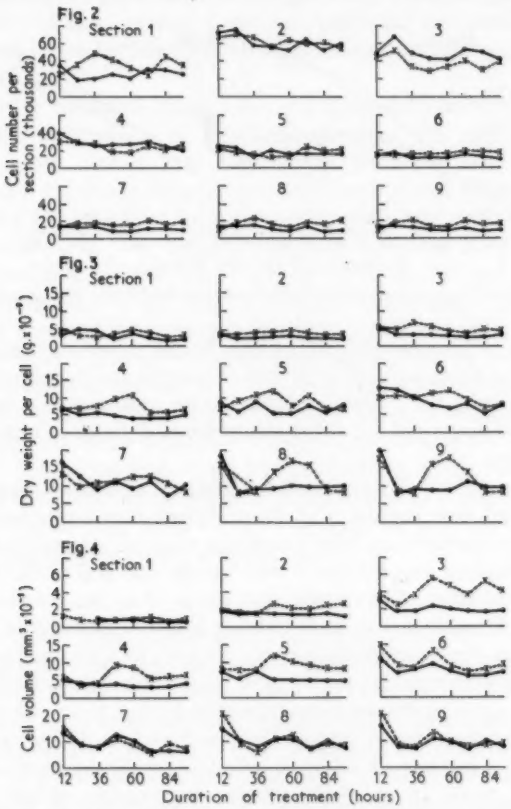
FIGS. 1*a–b*. Effect of boron and sugar on radicle growth of field bean. B = Boron, S = Sucrose.

was supplied for 7 hours only in each day. It had previously been found that supplying sugar for a 24-hour period in these non-sterile cultures depressed the growth of even the plus-boron roots. The results clearly indicated that the presence of sugar did not delay the onset of deficiency symptoms under these conditions.

(*c*) *Effect on cell number per section.* Estimates of cell number per section (Fig. 2) showed that section 3 of the deficient root contained fewer cells than that of the normal root. This result was surprising in view of the swollen nature of a deficient root tip but it was known that cell division had ceased so that an explanation of the swelling in terms of an increase in cell volume rather than cell number was, in fact, more likely. The lowered cell number of section 3 was due to an expansion of cells both longitudinally and radially, since root diameter was also found to increase. In sections further from the apex deficient roots contained more cells than normal roots due to the formation of the abortive lateral initials discussed above. These regions of the root contained a mixture of enlarged cells and smaller cells which were part of the lateral roots. It was found, from examination of cell suspensions (Feulgen, aceto-carmine staining) macerated with pectinase, that the frequency of dividing cells in the apical meristem was 5–7 per cent. for the plus-boron series but only 0·5 per cent. in deficient roots after 48 hours. The frequency of dividing cells in sections 7–10 during the formation of the lateral initials was not determined; these roots did not develop beyond the primordial stage.

(*d*) *Effect on dry weight per cell, diameter of the section and cell volume.* The dry weight per cell (Fig. 3) was increased under conditions of deficiency except in sections 7–10 which showed a decreased weight per cell probably as a result of the presence of smaller cells in the abortive lateral initials formed in these regions.

The diameter of the sections was markedly increased by deficiency conditions and the volume of cells (Fig. 4) in the apex of deficient roots was also greater. The average cell volume in the distal sections which were composed of radicle and lateral initial cells was found to be similar to that of normal



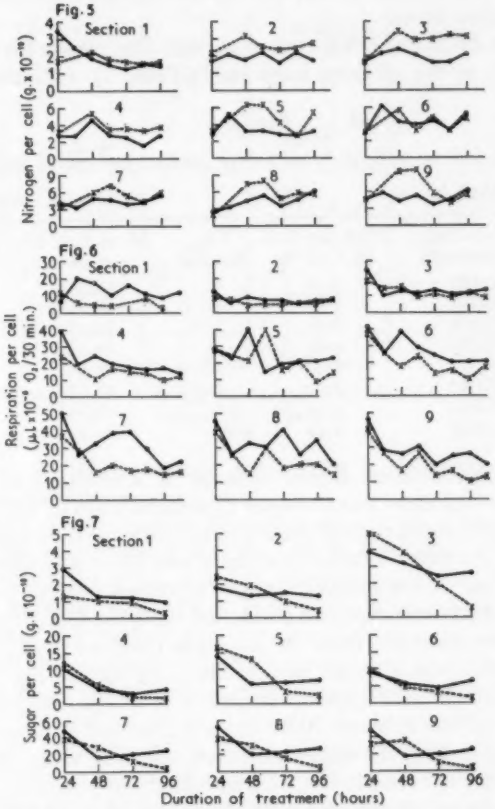
FIGS. 2–4. Effects of boron deficiency on (2) number of cells per 1 mm. section of field bean radicle; (3) dry weight per cell g.$\times 10^{-9}$; (4) volume per cell mm.$^3 \times 10^{-9}$. (Note changes in scale.) The broken line with crosses indicates lack of boron and the continuous line with circles that boron was supplied.

roots. This may indicate that the cells formed before the cessation of mitosis by the apical meristem of the deficient root reached a larger final volume than normal cells since the lateral initial cells would not be expected to be fully grown.

(*e*) *Effect on nitrogen content, respiration rate, and sugar content per cell.* As may be expected, the values for total nitrogen (Fig. 5) followed the estimates for cell volume and showed particularly the increased nitrogen content

of the cells from section 3. The effect was also apparent in sections 9–10 where the mean cell volume was not greater in deficient tissue.

Respiration rates per cell (Fig. 6) were generally lowered by deficiency although in sections 2–3 the values obtained were similar for deficient and normal tissue. The disparity in volume per cell between the treatments was



FIGS. 5–7. Effect of boron deficiency on (5) total nitrogen content per cell of field bean radicle; (6) respiration rate per cell; (7) sugar content (expressed as reducing sugar) per cell of field bean radicle. Legend as in Figs. 2–4. Note changes in scale.

greater in section 3, in which the respiration rate per unit volume was only 50 per cent. that of a cell of similar volume from a normal root.

The results of the sugar analyses were of particular interest in relation to the hypothesis relating boron deficiency to sugar translocation. The results (Fig. 7) suggest that total sugar was not decreased in the meristem until after 48 hours of treatment, by which time growth had slowed in deficient

roots and enlargement of the cells of section 3 was already marked. Certainly at the 96-hour stage the cells of deficient roots, irrespective of their location, contained less sugar than those of the plus-boron series. Considerable differentiation had, however, occurred in the deficient roots and xylem elements were found in section 2 which normally contains only meristematic cells. Sommer and Sorokin (1928) also reported xylem elements in this region of boron-deficient roots.

(*f*) *Effect on RNA and DNA content per cell.* The results for DNA content per cell, made at the 96-hour stage only (Table I), indicated that it was

TABLE I

Effect of boron deficiency on DNA content per cell in field bean

With boron			Without boron		
Section	Mean Cell No. per section	DNA per Cell [g. × 10^{-11}]	Section	Mean Cell No. per section	DNA per Cell [g. × 10^{-11}]
1	34,161	3·95	1	36,516	4·58
2	59,806	3·88	2	66,894	4·54
3	27,160	2·94	3	43,560	4·30
4	10,403	4·43	4	23,046	5·17
5	10,742	2·95	5	18,788	4·89
6	8,947	2·30	6	22,922	3·53
7–8	8,839	3·54	7–8	16,751	5·68
9–10	6,921	4·44	9–10	15,313	6·12

unlikely that cell division ceased because of a shortage of this nuclear component. In fact there was evidence of accumulation of DNA in deficient roots. Examination of deficient tissue showed that its cells had large diffuse nuclei and in the meristematic zone there was often a tendency to form a distorted nucleus with a partially extruded portion related, perhaps, to the nucleolus. There is evidence that in normal roots the DNA content per cell rises as distance increases from the root apex (Holmes *et al.*, 1955) and the apparently higher values found in deficient roots may be due to comparing tissues of different physiological development. It will be noted in these results that section 3 of the deficient roots had a higher cell number than normal roots. This contradicts the earlier estimates but even when this higher cell number per section is used to calculate the mean cell volume the latter is still found to be greater in the deficient root. Estimates of DNA and RNA content were also made on unsectioned centimetre portions at the 96-hour stage (Table II). The cell numbers calculated per section in the first DNA

TABLE II

Effect of boron deficiency on DNA and RNA content per cell in field bean

	With boron	Without boron
RNA per cell [g. × 10^{-11}]	9·31	7·73
DNA per cell [g. × 10^{-11}]	4·45	4·67

determination were used to express these values on a per cell basis. The results again showed no diminution of DNA per cell with deficiency although the accumulation was less marked. The RNA content per cell was evidently reduced under deficiency conditions but because this nucleic acid is not related solely to the nucleus it is difficult to know how the RNA relates to

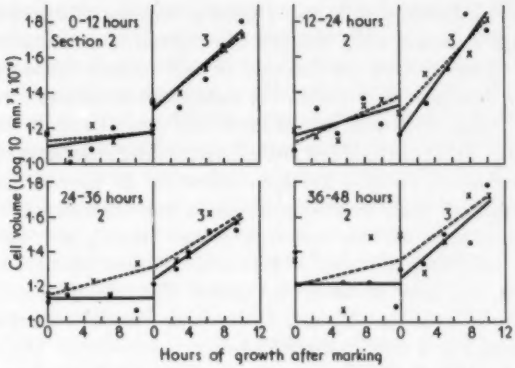


FIG. 8. Effect of boron deficiency on elongation of cells of field bean radicle. Legend as in Fig. 2–4.

the cell volume in deficient tissue, particularly as this component was not determined in the different regions.

(*g*) *The effect of deficiency on elongation of cells.* In a separate series of experiments the elongation of root tip cells was followed. The roots were grown in one-pound jam jars at 25° C. and the second or third section was delimited by marking the root with indian ink on transfer to and after 12, 24, and 36 hours of growth in plus and minus-boron nutrient solution. Ten of the 50 roots thus marked in each treatment were used to determine the initial cell volume. Further groups of 10 roots were harvested at convenient intervals over the next 10 hours. The cell volume was estimated from the measurement of diameter, length of the marked segment at harvest and cell number per segment estimated as previously described. A single estimate was made on the development of section 2 and two on that of section 3. The results (Fig. 8) use the mean values for the two experiments on section 3 and are presented together with those of section 2 to aid comparison, although they were obtained at different times. The cell volumes showed that, at the start of the experimental period, the cells of section 3 in deficient roots were larger than those of a normal root but that thereafter they grew more slowly giving a final cell volume similar to those of normal roots. This result agrees with the values obtained on cell volumes earlier. There appears to be no evidence that the cells of the deficient root enlarge unusually rapidly throughout their development although they may do so during the first stages of elongation. Cell division, however, is decreasing in frequency in the

meristem and the increased volume of deficient cells may be explained simply by their greater age at the time of marking.

DISCUSSION

The first effects of boron deficiency during the growth of the bean root were apparent in the meristematic zone. These symptoms were a decrease in the number of dividing cells and a greater volume of the cells at the root apex. Although deficient cells might have expanded unusually rapidly after initiation it is possible that the increase of cell volume followed as a consequence of the decrease in division. As mitosis became less frequent in the meristematic region each cell would have had more time in which to grow before it became a cell of the third and of succeeding segments. In particular, after the cessation of division (at approximately 48 hours) the apical cells would have retained their positions and have been compared with the more recently formed cells of non-deficient roots. During the early stages of deficiency the cells appeared to elongate normally and reached the same size as normal cells, but after division had ceased the cells of the deficient apex did not appear to elongate further. This was possibly a secondary effect due to the absence of a terminal meristem.

The cessation of mitosis may be a consequence of an effect on cell wall metabolism as a result of which the cell is prevented from becoming organized for division. During this process the cells may enlarge abnormally rapidly but this is not an essential requirement of a theory relating failure of division to an initial effect on the cell wall. Alternatively the cells may fail to divide because deficiency has a direct affect on the components of the nucleus or cytoplasm. The latter view is favoured by Gauch and Dugger (1953), Sisler *et al.* (1956), and Dugger *et al.* (1957) who suggested that division ceased as a result of lack of sugar due to the need for boron in translocation. It has been suggested (Whittington, 1957) that this evidence is not conclusive because of the variability in the estimates of translocation rate on different days and the possible relationships between the rates of utilization and transport of sugar. The results presented here and those of McIlrath *et al.* (1955) and Sisler *et al.* (1956) showed that meristems lacking boron were not restored to normal activity by an external supply of sugar. There was no evidence in any of these experiments of the amount of sugar absorbed; the results would be expected if boron were concerned in the initial movement of sugar into the plant.

Although White-Stevens (1938) reported that boron-deficient meristems were lacking in sugar, Odhnoff (1957) found that sugars accumulated in deficient roots. Moreover, McIlrath and Palser (1956) reported that carbohydrates were translocated in boron-deficient plants until the phloem became necrotic. The estimates for sugar content made as deficiency developed (Fig. 7) showed that there was accumulation of sugar in the cells of deficient meristems at 24 and 48 hours but a marked reduction per unit volume at the latter stage. The sugar content per cell at the apex fell markedly

over the later time periods and the deficient cells contained less sugar per unit volume than cells of similar size from normal roots. There was certainly an alteration in the pattern of sugar distribution in the deficient root but it appears to be a consequence rather than a cause of the cessation of cell division at the apex. Cell division had ceased by 48 hours. The lower sugar content in these and elongating cells was not evident until this stage and possibly resulted from the utilization of available supplies of sugar by the numerous lateral initials developing close to the apex of the deficient root. It is also likely that cell elongation requires more sugar than division, yet there is no evidence that cell elongation is affected until after the 48-hour stage.

The estimates of DNA and RNA showed that the content per cell was not greatly affected and the total nitrogen was directly related to the cell volumes. The evidence that boron deficiency is not concerned with DNA formation was not absolute. Assuming that its rate of synthesis is reduced by deficiency the apical cells may have differentiated, thus rendering further division unlikely, before sufficient DNA for mitosis had been formed within the cells. Because DNA was only determined at the 96-hour stage, when the apex was occupied by enlarged cells, sufficient time may have elapsed for the formation of the remaining DNA. Extensive observations have not revealed the presence of polyploid cells nor specific chromosome or spindle aberrations. There is, therefore, no evidence that lack of boron is the direct cause of an upset in the components of the nucleus or cytoplasm of the meristematic cells.

Boron deficiency may then exert some influence on the walls of meristematic cells such that the necessary environment for mitosis is not achieved. An attempt to determine if this process was accompanied by an alteration in the rates of expansion of meristematic cells during the early stages of elongation was not sufficiently precise to separate this possibility from the effect on cell volume of fewer cell divisions in the apex. A relationship between boron and the formation of cell walls, including particularly pectic substances has frequently been suggested (cf. Marsh and Shive, 1941; Winfield, 1945). Quantitative estimations of wall components in boron-deficient roots (Odhnoff, 1957) were interpreted as showing that both pectic substances and cellulose accumulated under deficiency conditions. These estimations were made on whole root systems and gave no information as to the changes that occur in any one region as deficiency progresses. Furthermore, three of the groups of plants analysed were of similar age but showed marked variation in the amount of pectic substances present. This evidence would appear to be inadequate to exclude the possibility of boron being of importance in the actual synthesis of cell-wall material.

The only single cells that have been studied during development in boron-deficient media are pollen grains; there is a wealth of evidence (cf. Gauch and Dugger, 1954) that demonstrates boron to be essential for their germination. The grains in the absence of boron either burst without germination or produce distorted pollen tubes which may also burst. Only one nuclear

division occurs during germination but there is no evidence as to whether this fails to take place in born-deficient media. The bursting and distortion appear to be related to an inability to form the pollen tube wall.

Both Odhnoff (1957) and Torssell (1956), who investigated the action of organic boron compounds on the growth of roots, believed that boron is concerned in the incorporation of substances into the cell wall and they suggested that deficient cells were unable to elongate. This is not supported by the present results. It seems likely that the rapid 'stiffening' of the cell wall due to cellulose deposition, mentioned by Torssell, occurs after the cell has enlarged completely. Spurr (1957) has concluded that the level of boron in the plant affects the rate of carbohydrate condensation into wall material. He showed that whereas collenchyma had thin walls under deficiency conditions, phloem parenchyma, and ground parenchyma in the same sections had thicker walls than usual. He interpreted this as being due to the closer proximity to phloem, the source of available sugar. It is known, however, that pectic substances are present in marked quantity in collenchyma, possibly alternating with layers rich in cellulose (Esau, 1953). As pectins, cellulose, and hemicelluloses are thought to arise by different pathways from the common precursor uridinediphosphate-D-glucose (Altermatt and Neish, 1956), the results obtained by Spurr are such as would be expected from a blockage in the path of pectin synthesis.

There is then a substantial body of indirect evidence which suggests that boron is associated with the synthesis of pectic compounds and is rendered immobile by complexing with it during synthesis. Thus it is possible that pectin synthesis ceases in the absence of continued supplies of boron, whereas cellulose formation is maintained or enhanced. Since pectin appears to be of particular importance in the young cell and does not increase directly with cell volume as does cellulose (Fuller, 1958) the evidence indicating that any alteration of the rate of elongation in the deficient root was in the earliest moments of cell expansion affords some support for this theory. On the other hand, the absence of polyploid cells in the apex suggests that middle lamellae are still formed in newly arisen cells. There is little information on the detailed structure of the walls of meristematic cells and the significance of their pectin components so that the above hypothesis must be regarded as tentative. Yet if study of the effect of boron on the pectin components of meristematic and elongated cells proves the theory untenable, it may be necessary to seek for an explanation of the role of boron in the complex organization which determines whether a newly formed cell will divide again or elongate.

It is a pleasure to acknowledge the helpful advice and criticism of Professor F. L. Milthorpe during the preparation of this paper. I should also like to thank Dr. K. W. Fuller, Department of Biochemistry, University of Sheffield, for several fruitful discussions and suggestions on the role of boron, Dr. J. K. Heyes for the analysis of the nucleic acids, and Mr. L. Heathcote for his technical assistance.

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The Effect of Length of Dark Period on the Amplitude of Leaf Movement in *Bauhinia monandra*

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Received 30 June 1958

SUMMARY

In *Bauhinia monandra*, a short-day plant with a critical daylength for flowering of about 12 hours, there is a progressive decrease in the amount of nastic leaf movement as daylength increases. When the daylength is increased from 18 to 18·5 hours, however, there is such a sudden decrease as to suggest the existence of a critical dark period at that daylength. The parallel with the critical dark period in the photoperiodism of some short-day plants lends some support to Bünning's endogenous rhythm explanation of photoperiodism. All the same, in *Bauhinia* the same rhythm cannot very well control both leaf movement and photoperiodic response, for the observed critical daylengths do not agree.

INTRODUCTION

THE effects of daylength on plants, including effects on flowering, have been explained by Bünning (1944, 1947, 1956), in terms of the inherent internal rhythm of the plant. He has often quoted sleep movements of leaves as excellent indications of endogenous rhythm.

From an analysis of the effects of daylength on the leaf movements of *Bauhinia monandra* (a tropical tree of the Caesalpiniaceae) I have, however, come to the conclusion (Holdsworth, 1959) that if internal rhythms do produce photoperiodic responses, then in *Bauhinia monandra* there must be a quite separate rhythm regulating its leaf movements: Bünning supposes that a daylength which prevents flowering is one which suppresses the rhythm (prevents the attainment of its 'photophile and scotophile maxima') yet in *Bauhinia*, daylength appeared to have little effect on the amount of movement of leaves unless it was some 6 or 7 hours greater than is critical for flowering of this plant.

Unfortunately, on this particular point, that is the effect of day-length on amplitude of the rhythm, my earlier data left some room for doubt. I have therefore now made more critical measurements of the effect on larger numbers of plants and over a greater range of daylength.

METHODS

Seedlings were raised in natural daylight until the first foliage leaf was a week old. They were then given one dark and one light period imitating the natural day of 12 hours. Then followed the experimental dark period which

always began at 6 p.m. Subsequently, the plants were kept in continuous light while their leaf movements were recorded by photography. On germination the seedlings were pricked out singly into pots so that each could be arranged to present its leaf in profile to the camera. Each plant was cut off above the first leaf before use, so that other leaves would not interfere with the camera's view. Illumination for each 10 plants came from two 150-watt reflector lamps screened by 3 cm. of water in a perspex trough. During the dark periods the plants were covered with thick black cloth. Negatives (35 mm.) were projected through a microfilm reader for measurement of the leaf angles. The plants were photographed immediately before and after the experimental dark period; once when they were fully open in the light (to obtain the 'maximum' angles which are given in Table I), and at quarter-hour intervals as they next approached the closed position (the 'minimum' angles in Table I): in continuous light the leaves remain open for several hours; but on closing, after reaching the minimum angle, they reopen at once.

TABLE I

The effect of length of dark period on the angles (degrees) attained by the leaves of Bauhinia, during the open and closed phases of the movement cycle. All dark periods began at 18.00 hours

Where more than one datum is given, they are from treatments repeated on separate occasions.

Darkness ended	Maximum angle	Minimum angle	Hour of reaching minimum	Number of hours illumination before minimum reached
—	78·8	69·5	17.45	23·75†
19.00	80·5	68·9	17.30	22·50
20.00	79·7	68·0	15.00	19·00
21.00	106·2	86·2	14.15	17·25
	91·0	76·2	14.30	
22.00	94·8	71·3	15.15	17·25
	91·8	70·4		
23.00	95·6	66·2	15.15	16·25
	89·2	69·4		
23.30	101·9	58·0	14.45	15·25
24.00	97·2	47·8	15.30	15·50
	97·1	50·4		
	93·7	46·3		
	91·2	42·5		
03.00	103·5	38·6	17.45	14·75
	98·5	42·5		
06.00	105·0	39·4	19.45	13·75
	104·8	39·0		
	103·4	38·2		
	103·4	37·9		
09.00	111·8	48·2	21.45	12·75
	104·0	27·9		
12.00	110·8	31·0	22.45	10·75
13.00	128·7	35·6	23.15	10·25
	116·0	29·7		
15.00	114·1	23·7	24.00	9·00
18.00	122·6	56·4	00.30	6·50

† i.e. the second closure in continuous light.

RESULTS

The time which elapsed in the light before the leaves closed, increased with decreasing length of the preceeding dark period (Table I). This confirms my previous results (loc. cit.) and can now be seen to be true for all periods of darkness from 1 to 24 hours. Comparing the hour at which closing occurred, however, it is seen to become earlier in the day as the dark period is reduced to 4 or 3 hours. With shorter periods still, it grew later again.

The data for the amplitude of movement (Table I and Fig. 1) need explanation: we are concerned to determine whether daylength affects the amplitude of a rhythm, using the amount of movement of leaves as an indication. Alternation of light and darkness, however, besides initiating diurnal leaf movements causes immediate leaf movement in *Bauhinia monandra*, for the leaves are photonastic. If then we are to observe a diurnal rhythm uncomplicated by

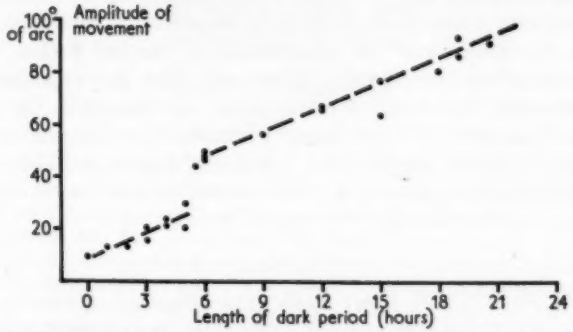

FIG. 1. Effect of length of dark period on the amplitude of movement of *Bauhinia* leaves.

photonastic movements, it must be observed either in continuous light or continuous darkness. If photography is to be used to record the movement, continuous light is the obvious choice. But daylength, the treatment to be tested, means alternation of light and darkness. Fortunately, it has been shown previously, that the leaves of *Bauhinia* will close in continuous light in response to a *previous* exposure to darkness. It is also fortunate that in a short-day plant like *Bauhinia*, the daylength effect on flowering is determined only by the length of the dark period. Bünning and his critics agree on this. It should be possible then to test the effect of daylength on the amplitude of leaf movement in *Bauhinia monandra* by measuring, in continuous light, the amount of movement as the leaves shut in response to the previous dark period.

The abscissae of Fig. 1 show the lengths of these dark periods. The ordinates show amplitudes of movement, i.e. the differences between the fully open and most nearly closed positions, and are averages from 10 leaves. The data from which the differences were calculated are given in Table I, and are measurements of the angle the leaf blade makes with the petiole. It can be seen in the table that both maximum and minimum angles were affected by length of dark period, the former increasing and the latter decreasing with longer dark periods. The difference between them is, however, seen in Fig. 1 not to increase steadily, but as the dark period is lengthened from 5 to 6 hours, there is a sudden increase in amplitude.

DISCUSSION

In the previous paper (Holdsworth, 1959) it was concluded that the critical daylength for the flowering of *Bauhinia monandra* cannot be much greater

than 12 hours. According to Bünning's theory of the mechanism of photoperiodism, we might expect a sudden decrease of the amplitude of the leaf movements of a short-day plant when the daily dark period is reduced below the critical length for flowering.

More detailed investigation of the relationship between dark period and leaf movement (Fig. 1) has confirmed that the sudden change occurs when the period is changed from 5 to 6 hours, but it is now seen that the amplitude goes on increasing as the dark period is lengthened further, even perhaps up to 24 hours. This extra observation could, of course, still be regarded as in agreement with Bünning's interpretation of daylength effects; for the effectiveness of photoperiodic induction in short-day plants usually does go on increasing as daylength is reduced below the critical. Flower initiation in many plants, for example, is progressively more rapid and more initials are laid down with increasing length of dark period (Lang, 1952).

However, the discrepancy, in *Bauhinia*, between the apparent critical dark periods for flowering and for leaf movement, leads to the conclusion that each must have its own rhythm, and that the two are out of phase. On the other hand, I have already (1959) pointed out that the diurnal movements of *Bauhinia* leaves can hardly be claimed to have an *endogenous* rhythm; for successive opening and closing movements fail to keep regular timing if alternations of light and darkness cease. In continuous light, for example, only the first closing movement occurs at the normal time. Subsequent movements are irregular and slight. I also found that the diurnal movements of *Bauhinia* leaves were almost unaffected by interruptions of the daily light period by dark periods even of several hours duration. The failure of leaf movement found here when the preceeding dark period was less than 5 hours may have little to do with suppression of a rhythm, and may simply be the effect of light *almost continuous from the morning before*. The slight closure that occurred following dark periods of less than 6 hours would then be equivalent to the second closure which occurs in a continuous period of illumination.

Leaf movement in continuous darkness. It has just been remarked that *Bauhinia* leaves kept in continuous light cease to open and close at regular intervals. The first closing movement occurs at the usual time but the leaves open again immediately afterwards, instead of remaining shut for several hours as they do with alternations of light and darkness. Some evidence was obtained in the earlier experiments (Holdsworth, 1959), that the timing of successive movements is abnormal in continuous darkness also. The new data confirm this conclusion, as can be seen in Fig. 2, where the leaf angles at the termination of the various dark periods have been collected together. In continuous darkness, opening begins at the usual time; i.e. after about 6 or 7 hours in the dark; it continues slowly throughout the following day. With 12-hour alternations of darkness and light, opening is completed soon after the end of the dark period. The experiments did not extend beyond a period of 24 hours in the dark, but it would appear that opening had only just ceased

by the end of that time. It seems that the effect of continuous darkness is complementary to that of continuous light. In the light, closure begins at the usual time, but reopening is accelerated; in the dark, opening begins at the usual time but its completion is delayed.

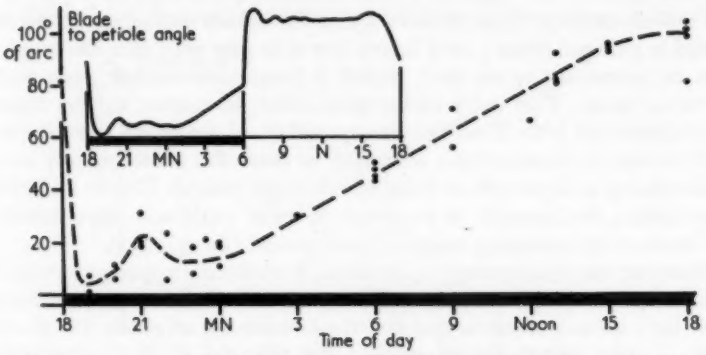

FIG. 2. Leaf movements of *Bauhinia* in continuous darkness, with (inset) a sketch of the usual cycle in 12-hour alternations of light and darkness.

These effects may be looked upon as consistent with the effects of alternation of light and darkness in a normal cycle where a change from darkness to light accelerates opening begun in the dark, and a change from light to darkness accelerates closure begun in the light.

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Effects of Mineral Salts on Short-term Incorporation of Carbon Dioxide in *Chlorella*[1]

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Received 27 July 1958

SUMMARY

The effects of various mineral salts have been studied, both singly and in combination, on the uptake and distribution of photosynthetically incorporated carbon-14 in *Chlorella*. When magnesium sulphate, potassium nitrate, and potassium phosphate were combined in the same concentrations as are found in the normal nutrient solution, the uptake of carbon-14 was increased and more radioactivity was incorporated into the amino-acids, with a decrease in the percentage of radioactivity in the sugar phosphates when compared to the controls in distilled water. The most striking effect of the addition of just one salt was obtained with salts containing nitrogen, particularly ammonium chloride, which in one experiment increased the radioactivity found in the amino-acids from 9·9 per cent. (expressed as percentage of the total soluble radioactivity found in this group of compounds) in the controls to 57 per cent., while the radioactivity in the sugar phosphates fell from 64 to 7 per cent., correspondingly. The total uptake of carbon dioxide by the cells supplied with ammonium chloride was three times as great as that in the controls in distilled water.

The relevance of these findings to the methodology involved in experiments dealing with the path of carbon in photosynthesis is discussed, with particular emphasis upon the concept of a 'steady state' and how it is thought this may best be achieved.

INTRODUCTION

ALTHOUGH the functions of the essential major elements in plant metabolism have been studied for many years, little work has been done concerning the effect of these elements during short-term incorporation of radioactive carbon dioxide. This may be of some importance as it has been the general custom during photosynthesis studies in this laboratory to suspend algae in various dilute buffer solutions (Bassham, Shibata, Steenberg, Bourdon, and Calvin, 1956; Barker, Bassham, Calvin, and Quarck, 1956) or in distilled water alone

[1] The work described in this paper was sponsored in part by the U.S. Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, California.

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(Lynch and Calvin, 1953; Norris, Norris, and Calvin, 1955), assuming that the salts remaining within the cells from the time of growth in nutrient solution are sufficient in quantity for the cells not to become deficient in one or more of the essential elements during the course of the experiment. There are some indications, however, that the addition of salts to algae suspended in distilled water may have a rapid, pronounced effect on some metabolic system within the plant. Thus, Clendenning, Brown, and Eyster (1956) have reported that *Nostoc muscorum*, if rinsed and resuspended in distilled water, loses most of its photosynthetic capacity, which can, however, be completely retored by the addition of potassium ion in concentrations no greater than a few parts per million. Also, K. Baalsrud (personal communication) found that the photosynthetic rate of a marine diatom, when suspended in synthetic magnesium-free water, can be greatly increased by the addition of magnesium salts. In view of these observations it appeared worth while to investigate the effects of the addition of the essential elements in those photosynthesis experiments in which the cells are kept in distilled water for varying periods of time.

In much of the work dealing with the path of carbon during photosynthesis (Benson, Kawaguchi, Hayes, and Calvin, 1952; Moses and Calvin, 1958*a*; Bassham, Benson, Kay, Harris, Wilson, and Calvin, 1953), the cells were suspended in distilled water and kept under constant conditions of temperature, lighting, and aeration for periods of up to 30 minutes or more before the radioactive carbon dioxide was introduced in order to achieve a 'steady internal metabolic state'. In experiments in which the adaptation time and the exposure time to ^{14}C are short, the possibility of a deficiency of one or more mineral elements affecting the pattern of ^{14}C incorporation is slight, but in longer-term experiments extending for an hour or more, the probability increases of such a deficiency occurring when cells are suspended solely in distilled water. This possibility of such a mineral deficiency would be of particular interest in those studies which purport to show some of the metabolic pathways of carbon not involved in the carbon cycle itself, such as the incorporation of carbon into pigment systems (Blass, 1957), or the interaction between the respiratory and photosynthetic pathways (Benson and Calvin, 1950; Calvin and Massini, 1952). This paper reports the effects of the major mineral elements, both singly and in combination, on the incorporation of radiocarbon in both light and dark in *Chlorella*. The effect of the ammonium ion, though not a constituent of the nutrient solution, has also been studied and its effects described.

METHODS

The alga *Chlorella pyrenoidosa* was grown in bacteria-free culture in steady-growth tubes under conditions previously described (Holm-Hansen, Hayes, and Smith, 1956). The composition of the nutrient medium was as follows: KNO_3, $1{\cdot}2\times10^{-2}$ M.; $MgSO_4$, $1{\cdot}0\times10^{-2}$ M.; KH_2PO_4, 8×10^{-3} M.; $Ca(NO_3)_2$, 1×10^{-4} M.; Fe (as the ferric-EDTA complex), 5 p.p.m.; B (as H_3BO_3) and Mn (as $MnCl_2$), 0·5 p.p.m. each; Zn (as $ZnSO_4$), 0·05 p.p.m.;

Cu (as $CuSO_4$), 0·02 p.p.m.; Mo (as MoO_3) and Co (as $CoCl_2$), 0·01 p.p.m. each. Samples were drained from the tubes and either pipetted into the small vessels used in the actual photosynthesis test, or centrifuged, rinsed, and resuspended in a variety of solutions prior to being pipetted into the small vessels. The construction and functioning of the apparatus which was used for the exposure of the algal cells to radiocarbon has been described elsewhere (Moses, 1957; Moses and Calvin, 1958*b*). The algal suspension (1·0 ml.) was contained in a cylindrical vessel which had a flat, optical-glass bottom and which was equipped with a glass top containing aeration and venting tubes; these flasks, which were partially submerged in a water bath, were mechanically shaken over a bank of fluorescent lights which illuminated the algal suspension with an intensity of approximately 2,000 f.c. The shaker held twelve such flasks, which enabled many parts of any one experiment to be performed simultaneously. After a certain period of adaptation of the shaker, during which time 1 per cent. (v/v) CO_2 in air was flushed through the vessels, any desired additions were pipetted directly into the algal suspension, following which 50 μl. of 0·026N. $NaH^{14}CO_3$ (400 μc/ml.) were added. After varying periods of photosynthesis with the labelled carbon, 4 ml. of boiling absolute ethanol were added to kill the cells. After extraction with 80 per cent. and 20 per cent. ethanol, the total insoluble cell material was centrifuged and discarded. The alcohol-soluble fractions were combined and concentrated by vacuum distillation at room temperature. Small samples of the extracts were then chromatographed as described by Benson, Bassham, Calvin, Goodale, Haas, and Stepka (1950) using phenol-water as the first solvent and *n*-butanol-propionic acid-water in the second direction. The locations on the paper of spots containing radioactivity were detected by autoradiography. The activity in each spot was counted by means of a Scott type Geiger-Müller tube with a thin (1 mg./cm.^{2}) 'Mylar' window. In experiments dealing with respiration or with the incorporation of $^{14}CO_2$ in the dark, the algal suspensions were contained either in Warburg vessels or in 10 ml. Erlenmeyer flasks and were shaken in the Warburg respirometer while in complete darkness. The killing procedure and subsequent manipulations of the extracts were the same as with samples in the light.

RESULTS

Nutrient solution and distilled water. The results from one typical experiment comparing the effect of distilled water and nutrient solution are shown in Table I. The total fixation of $^{14}CO_2$ by the algae in nutrient solution was increased by 89 per cent. over that in the controls with distilled water. The patterns of incorporation of ^{14}C seen on the developed chromatogram differed mainly in a decreased activity in the sugar phosphates in the nutrient solution sample (50 per cent. of the total soluble ^{14}C in the nutrient solution compared with 57 per cent. in distilled water), and a large increase in the activity incorporated into the amino-acids. It should be borne in mind that the figures for the incorporation of ^{14}C in the individual compounds are in terms of the

percentage of the total soluble ^{14}C in the extract which is present in that compound. Thus, when the total fixation is different in two samples, the percentage of activity in any one compound may be equal in both the samples,

TABLE I

Effect of nutrient solution on total uptake and distribution of carbon-14 during photosynthesis in Chlorella

1·0 ml. of 1 per cent. *Chlorella* suspension in distilled water. After 10 min. adaptation, 50 μl. of concentrated nutrient solution (final concentration equal to the usual growth medium) were added to the appropriate flasks and 50 μl. of water added to the controls. After another additional 10 min., the radioactive bicarbonate (20 μc.) was added and photosynthesis allowed to continue for 2 min. before addition of 4 ml. of boiling ethanol.

	Cells in distilled water	Cells in nutrient solution
Total fixation (dis./min.)	$9{\cdot}2\times10^6$	$17{\cdot}4\times10^6$
Total soluble activity (dis./min.)	$9{\cdot}0\times10^6$	$14{\cdot}4\times10^6$
Radioactivity in the following compounds as percentage of total soluble activity:		
Diphosphates	15·2	3·3
Uridine diphosphoglucose	2·1	3·5
Hexose monophosphates	30·1	35·6
Phosphoglyceric acid	6·5	4·3
Glucose cyclic phosphate	2·1	2·0
Sucrose	27·2	19·8
Alanine	4·6	15·1
Aspartic acid	2·2	5·0
Serine and glycine	3·7	2·7
Citrulline	—	0·01
Glutamic acid	—	0·07
Malic acid	3·5	—
Glycolic acid	0·1	0·85
Unknown 1, probably amino-acid	0·5	0·88
„ 2, „ organic acid	0·8	2·3
„ 3, „ „	—	—
„ 4, „ amino-acid	—	2·8
„ 5, „ sugar phosphate	1·1	0·95
„ 6, „ „ „	0·3	—
Sugar phosphates as percentage of total soluble radioactivity	57·4	49·8
Amino-acids as percentage of total soluble radioactivity	11·0	26·5

though the absolute amounts may vary considerably. Thus, the sugar phosphates decreased in percentage of the total activity fixed, but the absolute amount increased from 5×10^6 dis./min. to $7{\cdot}2\times10^6$ dis./min.

To determine which of the salts in the nutrient medium was having an enhancing effect on the fixation, and also which were affecting the distribution patterns of the carbon, the same type of experiment was carried out with the individual salts of the nutrient solution. The micro-elements boron, manganese, molybdenum, zinc, copper, cobalt, and also iron and calcium, were

not further examined as it was considered extremely unlikely that a deficiency for any one of these elements could be elicited by the procedures used. The results are shown in Table II. It is seen that the fixation in nutrient solution

TABLE II

Effect of the individual salts of the nutrient solution on the total uptake and distribution of carbon-14 during photosynthesis in Chlorella

1·0 ml. of 1 per cent. *Chlorella* suspension in distilled water. After 10 min. adaptation time on the shaker, 50 µl. aliquots of the appropriate solutions were added and the adaptation continued for another 10 min. The radioactive bicarbonate (20 µc.) was then added, followed by 4 ml. of boiling ethanol after 2 min. The pH of the added salt solutions was 5·3 in all cases.

	Cell suspension medium					
	Distilled water	0·012 M. KNO_3	0·008 M. KH_2PO_4	0·012 M. KNO_3 + 0·008 M. KH_2PO_4	0·01 M. $MgSO_4$	Nutrient solution
Total fixation (dis./min. × 10^{-6})	5·15	5·70	7·40	8·13	7·62	7·47
Total soluble radioactivity (dis./min. × 10^{-6})	4·07	4·50	6·28	7·03	6·55	6·10
Total radioactivity (as per cent. of total soluble radioactivity)						
Diphosphates	22·4	23·3	14·5	15·3	16·5	22·7
Uridine diphosphoglucose	2·4	1·8	2·5	1·7	2·6	2·0
Hexose monophosphates	37·1	28·6	48·6	40·3	36·6	21·0
Phosphoglyceric acid	9·3	9·5	14·5	14·3	14·0	15·5
Glucose cyclic phosphate	2·0	—	1·2	0·94	0·96	1·0
Sucrose	9·3	9·6	12·3	14·0	13·7	17·9
Alanine	2·9	7·0	2·3	5·2	5·7	6·9
Serine	1·3	2·1	1·0	1·3	1·2	1·8
Aspartic acid	0·56	2·6	0·24	1·6	1·6	1·9
Glycine	0·59	1·1	0·31	0·34	0·60	—
Malic acid	2·4	7·5	1·2	2·9	4·1	3·6
Glycolic acid	0·52	1·6	0·35	0·16	0·34	0·55
Fumaric acid	3·9	1·5	—	0·50	0·73	0·59
Glutamic acid	—	—	—	0·68	0·61	0·88
Unknown 1, probably sugar phosphate	0·23	2·5	0·18	0·34	0·23	2·7
Unknown 2, probably sugar phosphate	4·2	0·26	0·69	0·60	0·50	0·67
Sugar phosphates (as percentage of total soluble radioactivity)	77·6	66·0	82·2	73·5	71·4	65·8
Amino-acids (as percentage of total soluble radioactivity	5·4	12·8	3·9	9·1	9·7	11·5

was significantly higher than that in distilled water (increased by 45 per cent.), and that the separate additions of potassium nitrate, potassium dihydrogen phosphate, and magnesium sulphate all increased the fixation, the latter two causing approximately the same fixation as the complete nutrient solution. The figures showing the percentage of the activity incorporated into the sugar phosphates and into the amino-acids are interesting in that, compared with the controls, the addition of potassium nitrate increased the activity in the amino-acids and decreased it in the sugar phosphates, while the addition of potassium dihydrogen phosphate had just the reverse effect, i.e. the activity in the sugar phosphates was high while the activity in the amino-acids was decreased. Other experiments using potassium chloride, sodium nitrate, and sodium dihydrogen phosphate indicated that the potassium ion was without effect, and that the nitrate and phosphate groups were responsible for the observed effects. It is not surprising that responses were elicited by the

addition of nitrogen and phosphorus salts, as these are usually the first elements which apparently become limiting for algal growth both in the laboratory and in their natural habitat of lakes and streams (Gerloff and Skoog, 1954). If the cells are short in their supply of nitrogen and phosphorus, the effects noticed are just those which would be expected to occur. As in the first experiment, the nutrient solution caused a shift in the radioactivity from the sugar phosphates to the amino-acids.

Effect of ammonia. If a deficiency of nitrogen is limiting the conversion of sugar phosphates to amino-acids, &c., then it might be expected that ammonium ion would have a much greater effect in these short-term experiments than nitrate, due both to its more rapid penetration into the algal cells and to the fact that it already is reduced and can be utilized directly in amination reactions (Syrett, 1956). Many preliminary experiments bore out the suggestion that ammonium nitrate might have a marked effect on the fixation of carbon. In Table III is shown the effect of ammonium nitrate (0·001 M.) on

TABLE III

Effect of varying the length of the adaptation period with ammonium nitrate on total uptake of carbon-14 during photosynthesis in Chlorella

3·0 ml. of 1 per cent. algal suspension. Photosynthesis time in the presence of $^{14}CO_2$, 30 sec. Adaptation period before injection of $^{14}CO_2$, 30 min. Final concentration of ammonium nitrate, 0·001 M. 50 μl. $NaH^{14}CO_3$ added (20 μc.). Total time elapsed between harvesting and killing, 2 hrs.

	Total fixation (dis./min. $\times 10^{-3}$)	Soluble activity (per cent. of total fixed)
A. Control (algae in distilled water) . . .	2,800	90
B. $^{14}CO_2$ plus NH_4NO_3 injected simultaneously.	3,540	90
C. NH_4NO_3 adaptation period of 30 min. . .	4,070	91
D. Cells in NH_4NO_3 during all rinsings, &c. .	4,770	88

the total fixation of carbon after the cells have been exposed to the ammonium nitrate for varying periods of time before the addition of the radioactive bicarbonate. The time of exposure to the ^{14}C was the same in all cases, namely 30 seconds. The longer the period of contact with ammonium nitrate before the addition of the ^{14}C, the greater was the amount of carbon dioxide which was fixed; ammonium nitrate still had a noticeable effect on the total fixation and pattern of ^{14}C distribution even when added simultaneously with the bicarbonate.

To separate out the effects of nitrate and ammonium ions, the effect of potassium nitrate and ammonium chloride were studied individually. The data presented in Table IV and Fig. 1 show the effects of nitrate and ammonium ions on the total fixation and distribution of the ^{14}C compared with algae in distilled water. Ammonium ion increased the total fixation almost threefold, while nitrate did not increase it significantly. The distribution of the carbon (Fig. 1) was markedly affected in the cells supplied with ammonium ion, as can be seen from the percentage of the total fixed activity incorporated into

TABLE IV

Effect of potassium nitrate and ammonium chloride on total uptake and distribution of carbon-14 during photosynthesis in Chlorella

1·0 ml. of 1 per cent. *Chlorella* suspension. Photosynthesis time in presence of ^{14}C, 2 min. Cells originally in distilled water and adapted on the shaker for 10 min., at which time 50 μl. of distilled water (as control), NH_4Cl solution (final concentration in algal suspension, 0·002 M.), or KNO_3 solution (final concentration in algal suspension, 0·002 M.) were pipetted into the separate vessels. After a further period of 10 min. adaptation, the radioactive bicarbonate (20 μc.) was added. The cells were killed by the addition of 4 ml. of boiling ethanol.

	Distilled water	0·002 M. KNO_3	0·002 M. NH_4Cl
Total fixation (dis./min. $\times 10^{-3}$)	6,700	7,000	18,500
Total soluble radioactivity (dis./min. $\times 10^{-3}$)	5,320	5,250	14,500
Sugar phosphates (as percentage of total soluble fixation)	64·4	58·6	7·1
Amino-acids (as percentage of total soluble fixation)	9·9	16·2	56·8

the sugar phosphates and into the amino-acids. The percentage of the total activity found in the early photosynthetic intermediates (sugar phosphates) decreased from 64 per cent. in distilled water to 7 per cent. when ammonia was present, while the activity in the amino-acids increased from 10 per cent. to 57 per cent. Nitrate had a similar, though much less marked effect.

Data showing the incorporation of carbon dioxide with varying times of exposure to the ^{14}C from 5 seconds to 3 minutes are presented in Table V. In this experiment the presence of ammonium ion again increased the total fixation of ^{14}C (by 5–10 per cent.), but not to the same extent as in previous experiments. There was no significant difference in the total fixation between the controls in distilled water and the cells suspended in nitrate. The distribution patterns of the incorporated ^{14}C were similar to those reported in previous experiments in that the addition of ammonium ion caused a greater percentage of the activity to be incorporated into the amino-acids at the expense of the sugar phosphates. The patterns of ^{14}C in the presence of nitrate ion seemed to be intermediate between the controls and ammonium samples as they too showed some shift of activity from the sugar phosphates into the amino-acids, but not nearly to the same extent as in the presence of ammonium ion. The ^{14}C content of the organic acids was also increased in the presence of added ammonium.

In order to determine whether the effect of ammonia on cells suspended in distilled water was due to the degree of nitrogen starvation, or to a more specific effect of ammonium ions *per se*, the incorporation patterns of $^{14}CO_2$ in the presence of ammonium chloride were examined in cells still suspended in the original nutrient solution, as well as in those in distilled water. Cells were removed from the culturing vessel and as rapidly as possible were placed in the photosynthesis vessels without any intervening centrifugation. Ammonium chloride was added to half the samples, and the cells photosynthesized in the presence of $^{14}CO_2$ for 2 minutes. Further aliquots of the cells from the culture

addition of nitrogen and phosphorus salts, as these are usually the first elements which apparently become limiting for algal growth both in the laboratory and in their natural habitat of lakes and streams (Gerloff and Skoog, 1954). If the cells are short in their supply of nitrogen and phosphorus, the effects noticed are just those which would be expected to occur. As in the first experiment, the nutrient solution caused a shift in the radioactivity from the sugar phosphates to the amino-acids.

Effect of ammonia. If a deficiency of nitrogen is limiting the conversion of sugar phosphates to amino-acids, &c., then it might be expected that ammonium ion would have a much greater effect in these short-term experiments than nitrate, due both to its more rapid penetration into the algal cells and to the fact that it already is reduced and can be utilized directly in amination reactions (Syrett, 1956). Many preliminary experiments bore out the suggestion that ammonium nitrate might have a marked effect on the fixation of carbon. In Table III is shown the effect of ammonium nitrate (0·001 M.) on

TABLE III

Effect of varying the length of the adaptation period with ammonium nitrate on total uptake of carbon-14 during photosynthesis in Chlorella

3·0 ml. of 1 per cent. algal suspension. Photosynthesis time in the presence of $^{14}CO_2$, 30 sec. Adaptation period before injection of $^{14}CO_2$, 30 min. Final concentration of ammonium nitrate, 0·001 M. 50 μl. $NaH^{14}CO_3$ added (20 μc.). Total time elapsed between harvesting and killing, 2 hrs.

	Total fixation (dis./min. $\times 10^{-2}$)	Soluble activity (per cent. of total fixed)
A. Control (algae in distilled water) . . .	2,800	90
B. $^{14}CO_2$ plus NH_4NO_3 injected simultaneously.	3,540	90
C. NH_4NO_3 adaptation period of 30 min. . .	4,070	91
D. Cells in NH_4NO_3 during all rinsings, &c. .	4,770	88

the total fixation of carbon after the cells have been exposed to the ammonium nitrate for varying periods of time before the addition of the radioactive bicarbonate. The time of exposure to the ^{14}C was the same in all cases, namely 30 seconds. The longer the period of contact with ammonium nitrate before the addition of the ^{14}C, the greater was the amount of carbon dioxide which was fixed; ammonium nitrate still had a noticeable effect on the total fixation and pattern of ^{14}C distribution even when added simultaneously with the bicarbonate.

To separate out the effects of nitrate and ammonium ions, the effect of potassium nitrate and ammonium chloride were studied individually. The data presented in Table IV and Fig. 1 show the effects of nitrate and ammonium ions on the total fixation and distribution of the ^{14}C compared with algae in distilled water. Ammonium ion increased the total fixation almost threefold, while nitrate did not increase it significantly. The distribution of the carbon (Fig. 1) was markedly affected in the cells supplied with ammonium ion, as can be seen from the percentage of the total fixed activity incorporated into

TABLE IV

Effect of potassium nitrate and ammonium chloride on total uptake and distribution of carbon-14 during photosynthesis in Chlorella

1·0 ml. of 1 per cent. *Chlorella* suspension. Photosynthesis time in presence of ^{14}C, 2 min. Cells originally in distilled water and adapted on the shaker for 10 min., at which time 50 μl. of distilled water (as control), NH_4Cl solution (final concentration in algal suspension, 0·002 M.), or KNO_3 solution (final concentration in algal suspension, 0·002 M.) were pipetted into the separate vessels. After a further period of 10 min. adaptation, the radioactive bicarbonate (20 μc.) was added. The cells were killed by the addition of 4 ml. of boiling ethanol.

	Distilled water	0·002 M. KNO_3	0·002 M. NH_4Cl
Total fixation (dis./min. $\times 10^{-3}$)	6,700	7,000	18,500
Total soluble radioactivity (dis./min. $\times 10^{-3}$)	5,320	5,250	14,500
Sugar phosphates (as percentage of total soluble fixation)	64·4	58·6	7·1
Amino-acids (as percentage of total soluble fixation)	9·9	16·2	56·8

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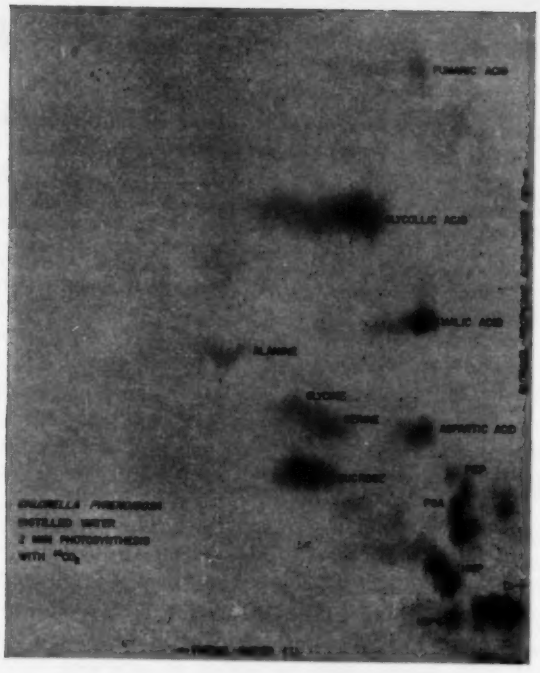
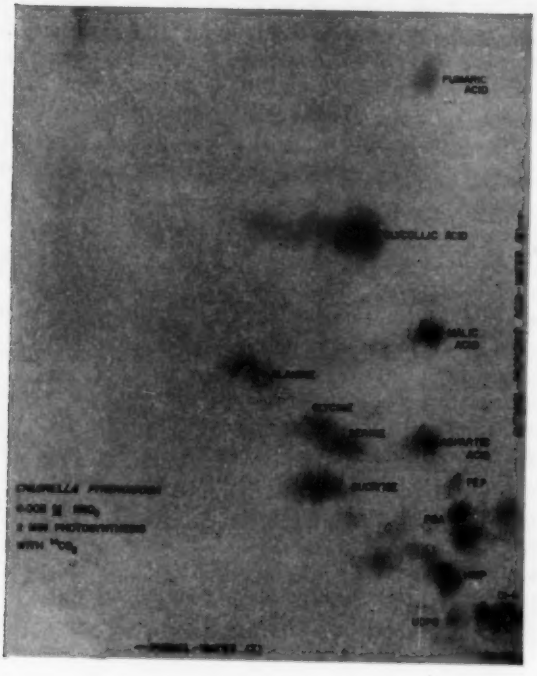
Data showing the incorporation of carbon dioxide with varying times of exposure to the ^{14}C from 5 seconds to 3 minutes are presented in Table V. In this experiment the presence of ammonium ion again increased the total fixation of ^{14}C (by 5–10 per cent.), but not to the same extent as in previous experiments. There was no significant difference in the total fixation between the controls in distilled water and the cells suspended in nitrate. The distribution patterns of the incorporated ^{14}C were similar to those reported in previous experiments in that the addition of ammonium ion caused a greater percentage of the activity to be incorporated into the amino-acids at the expense of the sugar phosphates. The patterns of ^{14}C in the presence of nitrate ion seemed to be intermediate between the controls and ammonium samples as they too showed some shift of activity from the sugar phosphates into the amino-acids, but not nearly to the same extent as in the presence of ammonium ion. The ^{14}C content of the organic acids was also increased in the presence of added ammonium.

In order to determine whether the effect of ammonia on cells suspended in distilled water was due to the degree of nitrogen starvation, or to a more specific effect of ammonium ions *per se*, the incorporation patterns of $^{14}CO_2$ in the presence of ammonium chloride were examined in cells still suspended in the original nutrient solution, as well as in those in distilled water. Cells were removed from the culturing vessel and as rapidly as possible were placed in the photosynthesis vessels without any intervening centrifugation. Ammonium chloride was added to half the samples, and the cells photosynthesized in the presence of $^{14}CO_2$ for 2 minutes. Further aliquots of the cells from the culture


FUMARIC ACID
GLYCOLLIC ACID
MALIC ACID
ALANINE
GLYCINE
ASPARTIC ACID
PGA
CHLORELLA PYRENOIDOSA
DISTILLED WATER
2 MIN PHOTOSYNTHESIS
WITH $^{14}CO_2$


FUMARIC ACID
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MALIC ACID
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ASPARTIC ACID
PEP
PGA
CHLORELLA PYRENOIDOSA
2 MIN PHOTOSYNTHESIS
WITH $^{14}CO_2$

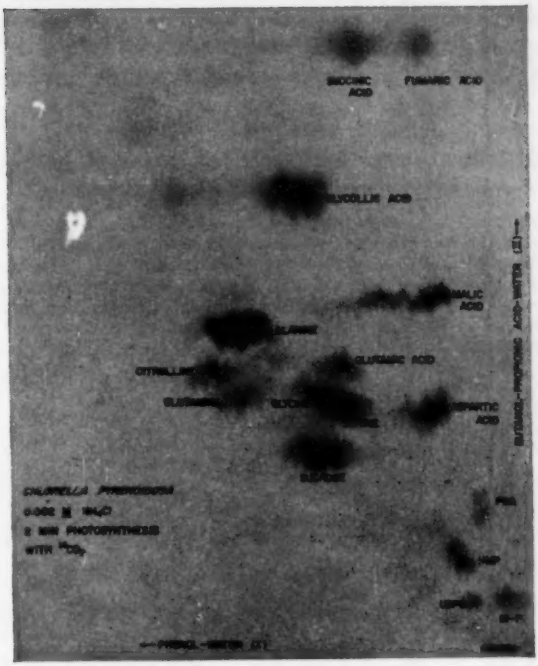

FIG. 1. Effect of potassium nitrate and ammonium chloride on the distribution of carbon-14 during photosynthesis in *Chlorella*.

For experimental details, see Table IV. Key to abbreviations: UDPG, uridine diphosphoglucose; Di-P, sugar diphosphates; HMP, sugar monophosphates; PEP, phosphoenolpyruvic acid; PGA, phosphoglyceric acid.

TABLE V

Effect of potassium nitrate and ammonium chloride on total uptake and distribution of carbon-14 after varying times of photosynthesis with labelled carbon dioxide

1·0 ml. of 2 per cent. *Chlorella* suspension in 0·067 M. phosphate buffer, pH 7·0. After 10 min. adaptation, 50 μl. of the appropriate solution (water, ammonium chloride, or potassium nitrate; final concentrations, 0·001 M.) were added, followed by another 12 min. of adaptation before the addition of the labelled bicarbonate (20 μc.). Photosynthesis times with the carbon-14 were as indicated below. The cells were killed by the addition of 4 ml. of boiling ethanol.

		Percentage of the total soluble radioactivity found in the more important compounds, together with the total activities in the various groups of substances												
		Totals in												
Incubation periods	Total fixation (dis./min. × 10^{-3})	sugar-P	amino-acids	organic acids	PGA	HMP	Di-P	UDPG	Sucrose	Alanine	Aspartate	Glutamate	Ser.+ Glyc.*	Citrulline
A. Controls														
5 sec.	84	92	—	7	73	12	7	—	—	—	—	—	—	—
10 sec.	195	94	—	7	54	29	11	—	—	—	—	—	—	—
30 sec.	908	92	3	4	30	47	15	—	—	1·7	0·1	—	1·1	—
60 sec.	1,800	90	5	4	24	50	14	1·1	0·3	3·6	0·2	—	0·9	—
120 sec.	2,920	84	7	4	17	53	11	2·8	2·5	3·3	0·6	—	2·6	—
180 sec.	3,900	79	9	5	14	47	14	3·8	4·7	4·3	1·2	0·2	2·8	—
B. With NH_4Cl														
5 sec.	79	81	—	18	46	29	6	—	—	—	—	—	—	—
10 sec.	214	74	7	19	35	31	8	—	—	2·0	—	—	—	2·9
30 sec.	970	74	12	12	22	41	11	—	1·0	6·0	1·9	—	—	4·2
60 sec.	1,920	60	24	13	18	32	9	1·2	2·9	12	3·2	—	1·6	4·0
120 sec.	3,330	50	33	13	10	29	10	15·0	2·3	17	6·0	3·0	—	6·0
180 sec.	4,600	42	44	14	7·0	24	9	2·0	0·7	22	5·0	4·0	3·0	4·0
C. With KNO_3														
5 sec.	126	97	—	3·5	68	21	8	—	—	—	—	—	—	—
10 sec.	246	92	—	3·7	51	28	13	—	—	—	—	—	—	—
30 sec.	870	92	4	3·0	29	47	16	—	—	2·2	0·5	—	1·2	—
60 sec.	1,530	88	7	4·0	22	48	17	0·9	0·4	4·0	1·0	—	1·0	—
120 sec.	2,920	80	13	5·0	17	45	16	3·0	2·2	7·6	2·6	0·6	1·6	—
180 sec.	3,900	76	14	5·0	14	42	17	3·0	4·8	8·0	3·0	1·0	2·0	—

* Not separated chromatographically.

Key: Sugar-P, sugar phosphates; PGA, phosphoglyceric acid; HMP, sugar monophosphates; Di-P, sugar diphosphates; UDPG, uridine diphosphoglucose; Ser., serine; Glyc., glycine.

vessel were centrifuged, washed, and resuspended in distilled water at the same concentration as those still in nutrient solution. After 7 minutes of pre-adaptation, these cells were also exposed to $^{14}CO_2$ for 2 minutes in the light in the presence and absence of ammonium chloride. Table VI shows that the

TABLE VI

Effect of ammonium ion on total uptake and distribution of carbon-14 by Chlorella cells suspended in distilled water and nutrient solution

1·0 ml. 0·8 per cent. *Chlorella* suspension. Suspension in nutrient solution was pipetted directly from the vessels in which the algae were grown, while the cells in distilled water were centrifuged and rinsed twice in distilled water before final resuspension in distilled water. After 7 min. adaptation, 50 μl. of 0·042 M. NH_4Cl (final concentration, 0·002 M.) were added to the appropriate flasks and 50 μl. of water added to the controls. After 5 more min., the radioactive bicarbonate (20 μc.) was added and photosynthesis allowed to continue for 2 min. before addition of 4 ml. of boiling alcohol.

	Cells in distilled water		Cells in nutrient solution	
	Controls	NH_4Cl	Controls	NH_4Cl
Total fixation (dis./min. $\times 10^{-4}$)	5·11	6·95	3·85	3·99
Total soluble activity (dis./min. $\times 10^{-4}$)	3·48	5·49	2·97	2·92
Radioactivity in the following compounds as percentage of total soluble activity:				
Phosphoglyceric acid	15·8	13·2	9·1	10·9
Diphosphates	8·5	13·0	4·1	4·6
Hexose monophosphates	53·3	28·6	45·8	43·2
Uridine diphosphoglucose	2·2	2·6	2·3	2·4
Malic acid	1·5	12·5	6·1	5·0
Citric acid	0·0	0·7	0·3	1·1
Sucrose	12·7	3·5	11·0	5·7
Aspartic acid	0·6	5·0	5·2	3·8
Glutamic acid	0·1	1·9	1·4	1·4
Serine and glycine	2·8	5·9	6·2	5·3
Alanine	2·5	13·2	8·2	16·6
Sugar phosphates (as percentage of total soluble activity)	79·8	57·4	61·3	61·1
Amino-acids (as percentage of total soluble activity)	6·0	26·0	21·0	27·1

cells remaining in nutrient medium demonstrated little change when ammonia was added, while those in distilled water exhibited contrasting patterns in the presence and absence of ammonium chloride typical for this situation (Table V). The effect of ammonia on the photosynthetic incorporation patterns of $^{14}CO_2$ is thus primarily a result of nitrogen deficiency in the cells, and not of a specific effect of the ammonia itself.

Dark fixation of carbon dioxide. There exists the possibility that the enhancement of the fixation of $^{14}CO_2$ by the addition of mineral salts may not reflect a photosynthetic uptake of carbon dioxide, but may merely be brought about by one or more dark reactions. To test this possibility, the effects of the various

glucose; Ser., serine; Glyc., glycine.

salts hitherto mentioned were examined for their effect on the dark fixation of radioactive bicarbonate. The results are shown in Table VII. It is seen that ammonium chloride increased the fixation about fourfold, while potassium

TABLE VII

Effect of mineral salts upon the dark fixation of carbon dioxide in Chlorella

5·0 ml. of 2 per cent. *Chlorella* suspension in distilled water in 25 ml. Erlenmeyer flasks. Shaken in complete darkness at 25° in air. After 15 min. the appropriate additions were made (250 μl. of each solution), followed by 30 min. of further adaptation, after which the radioactive bicarbonate (100 μC.) was added. After 4 min. exposure to the labelled carbon, the cells were killed by addition of 20 ml. of alcohol.

	Control	NH_4Cl (0·002 M.)	KH_2PO_4 (0·008 M.)	$MgSO_4$ (0·01 M.)	Nutrient solution
Total activity fixed (dis./min. $\times 10^{-3}$)	192	788	286	553	240

dihydrogen phosphate, magnesium sulphate and nutrient solution also increased it by varying amounts. Even though ammonium chloride increased the fixation four times the amount fixed was still relatively small compared with the usual light fixation value (5×10^6 dis./min./min. of incubation/ml. cells in the dark versus 900×10^6 dis./min./min. of photosynthesis/ml. cells). The quantitative aspects of this dark fixation invalidate the hypothesis that it is dark fixation reactions alone which account for the enhanced uptake of carbon dioxide in the light upon addition of the various salts. As the patterns of ^{14}C discussed hitherto reflect the interactions of the photosynthetic cycle and the respiratory mechanisms, it was also of importance to ascertain if the salts under consideration had any effect on the respiration rate of *Chlorella*. This was tested by the usual manometric methods (Umbreit, Burris, and Stauffer, 1949) and it was found that the addition of ammonium chloride did increase the dark respiration rate by about 80 per cent. (cf. Syrett, 1953), while nutrient solution increased it by about 40 per cent. Addition of potassium dihydrogen phosphate and magnesium sulphate apparently had no effect on respiration.

DISCUSSION

The results presented in this paper demonstrate that the total fixation of radioactive carbon dioxide, as well as the patterns of ^{14}C incorporation, by a suspension of *Chlorella* cells in distilled water may be altered by the addition of various mineral salts. When a readily assimilable nitrogen source such as ammonium chloride is added to the photosynthesizing cells, there is a greater incorporation of the labelled carbon into amino-acids, especially aspartate, glutamate, alanine, glutamine, and citrulline, and to a lesser degree, into the organic acids such as citric, fumaric, malic, &c. There is concomitantly a decrease in the ^{14}C usually found in the sugar phosphates and in sucrose. The interrelationships of some of the metabolic pathways involved here may be illustrated by Fig. 2. The addition of ammonium ion, and, to a lesser extent,

nitrate ion, accelerated the conversion of photosynthetic intermediates into other products as witnessed by the increase in the ^{14}C content of many of the organic acids and amino-acids which are involved in, or formed by, action of

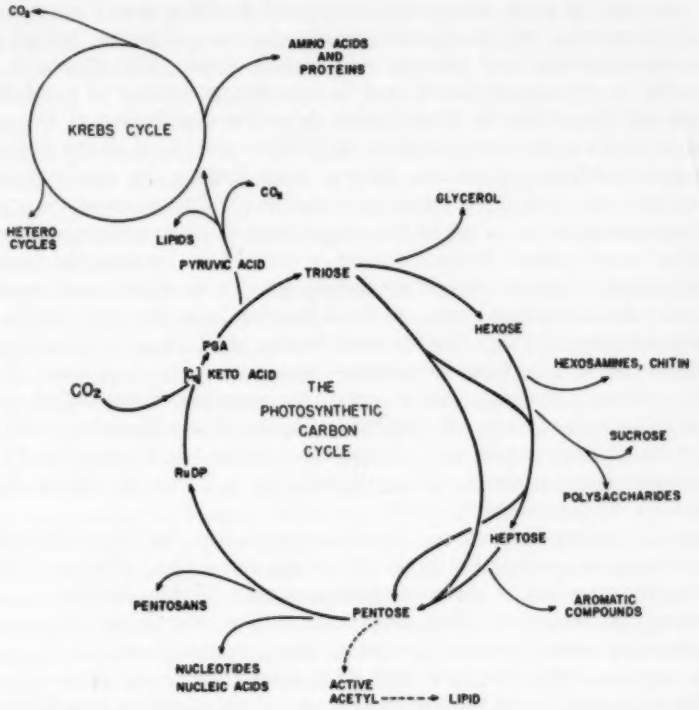

FIG. 2. Interrelationships between the carbon reduction cycle and other metabolic pathways.

the Krebs cycle. Although all the experiments that have been performed on this problem have consistently shown that addition of a nitrogen source increases the ^{14}C incorporated into many amino-acids, the quantitative results have not always been consistent with regard to an increase in total fixation, or even at times, in increasing significantly the activity in some of the organic acids. The causes of these fluctuations are not understood, but may reflect some unsuspected variation in the growing conditions of the algal cultures or in subsequent manipulation.

The possibility exists that the addition of these salts such as ammonium chloride will cause an alteration in the pH of the medium which may partially, at least, be responsible for both the apparent increased fixation and changes in the pattern of carbon dioxide incorporation. According to Ouellet and

Benson (1952), the pH region for maximum photosynthetic activity in *Scenedesmus* (and it can reasonably be assumed that the same will hold approximately true for *Chlorella*) was between 6·0 and 9·0, with decreased activity in either more acidic or more alkaline conditions. Further, at lower pH's (2·0–3·0), the main change in the distribution pattern of the ^{14}C was an increase in the activity in sucrose, polysaccharide material, alanine, and serine. At high pH's (10), incorporation into aspartic acid was favoured. The effects of salts discussed in this paper thus do not fit into the predictions of a pH effect, either with regard to the total fixation or to the distribution of the radiocarbon. In the experiment described in Table II the pH of all the added salt solutions had been adjusted to pH 5·3. After flushing the algal suspension with 1 per cent. CO_2-in-air for 10 minutes, the pH values ranged from 5·3 in the nutrient solution to 5·9 in the magnesium sulphate sample and in the distilled water control. Some elevation in the pH can be expected from the addition of the bicarbonate (pH about 8·8), but this should be small considering the volumes and molarities involved. Another point that warrants further mention is the possibility that the total fixation of ^{14}C is not indicative of the absolute rate upon different treatments within any one experiment. It may simply reflect differences in the size of the reservoir of unlabelled carbon dioxide due to changes in pH, ionic strength, &c. This will undoubtedly have some effect in the samples with ammonium chloride, but it cannot be of much importance in experiments such as those shown in Table III, where the pH values are all quite similar.

It has been regularly noticed in other experiments that when *Chlorella* or *Scenedesmus* were suspended in distilled water and the rate of photosynthesis continually measured in the steady-state apparatus (Wilson and Calvin, 1955; Bassham, Shibata, and Calvin, 1955), the rate of photosynthesis decreased about 5–10 per cent. per hour (personal communication from J. A. Bassham). This may mean that the cells were limited by a deficiency of one or more nutrient elements which resulted in a decreased rate of carbon dioxide uptake. The term 'steady-state', as employed in this laboratory (Calvin and Massini, 1952) means that the concentrations of the intermediates and enzymes involved in the carbon reduction cycle remain constant and do not change during the course of the experiment. There is thus no assumption inherent in this definition that all the other metabolic processes are in a 'steady-state' during the experiment. It is possible to conceive of changes occurring in some of the other metabolic pathways while the photosynthetic cycle itself is not materially influenced. Thus, the results presented in this paper show that some metabolic pathways may be influenced by the addition of salts, without greatly affecting others, particularly the pattern of compounds of the photosynthetic cycle. It is obvious that in time all photosynthetic activity will ultimately cease after the cells are placed in distilled water, but the time at which the first effect would be noticeable in the photosynthetic intermediates cannot easily be predicted from these results. It appears that there is no major change in the photosynthetic pattern in as long as an hour or more.

Thus, if one wished to obtain the best reproducibility from experiment to experiment, it would be advisable to eliminate this possibility of mineral-salt deficiency by suspending the cells in dilute nutrient medium of some sort. The addition of salts to the suspension complicates the subsequent chromatography, but we have found that, using the standard nutrient solution, one can place on the chromatogram origin at least 100 μl. of the nutrient medium. Using the amount of $^{14}CO_2$ commonly employed, the limitation caused by this volume of extract is not serious with photosynthesis times of half a minute or more, as there is enough fixed activity in samples of this size to give good radioautograms in a few days, or possibly a week or two. For short exposure times of a few seconds or less, the whole problem of mineral deficiency is not so serious, so that in these cases it might be advisable to use a medium of distilled water alone, as large aliquots of the extract must often be chromatographed.

If one were concerned about maintaining as steady a state as possible in the cells during a photosynthesis experiment, one of the best ways to minimize a change of conditions for the cells following their removal from the culture vessels would be to take them directly out of the growth tubes or flasks, pipette samples immediately into the small shaking vessels described previously, flush with the same aerating mixture as is used in all the growth vessels, and inject the labelled bicarbonate in the usual fashion. This technique could avoid the production of unknown transient phenomena which can be expected to occur if the suspension is flushed with air, nitrogen, &c., immediately before addition of the bicarbonate. This procedure would also eliminate the intervening centrifugations and other manipulations which are liable to have unknown effects on the metabolic activities.

The experiments described in this paper provide some insight into the capacity of *Chlorella* cells for the storage of such basic raw materials as nitrogen and phosphorus compounds. This capacity appears to be quite small, for within a short time (about 30 minutes) following the removal of the algal cells from the complete culture medium, signs of nitrogen (nitrate) and phosphorus (phosphate) deficiency can be observed when the cells are placed in conditions suitable for the rapid photosynthetic assimilation of carbon dioxide. Under optimal conditions newly assimilated carbon is presumably immediately converted to storage materials (polysaccharide) and to general cellular constituents, particularly protein, and the intracellular concentrations of soluble metabolic intermediates remains at a fairly low level. A deficiency of phosphorus, and especially of nitrogen, which are macro-constituents of cells, would limit many of the biosynthetic activities and lead to an accumulation of intermediates on the synthetic pathways, unless the assimilation of carbon dioxide were curtailed. Experiments have shown that both these effects can be demonstrated: when cells are suspended in media containing ammonium or nitrate ions there is an enhanced total fixation of $^{14}CO_2$ and a relative increase in amino-acids with a corresponding fall in the percentage of the activity in the sugar phosphates, compared with cells in distilled water.

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Respiration Rate and Mitochondrial Oxidase Activity in *Arum* Spadix

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Received 11 August 1958

SUMMARY

1. Mitochondria prepared from young *Arum* spadix oxidise succinic and α-ketoglutaric acids at about 300μl. O_2/hr./g. wet wt. but as the spadix ages the activity of the mitochondria increases more than tenfold. The increase results from a small rise in the quantity of mitochondrial nitrogen brought down in the centrifuge and a larger rise in oxidase activity per mg. mitochondrial nitrogen.

2. The rate of respiration of spadix slices has been measured under conditions in which it is not limited by slow diffusion of oxygen. Slices of young spadix respire at about 1,000–2,000 μl. O_2/hr./g. but as the inflorescence opens the rate soars to 20,000μl. O_2/hr./g. and the spadix becomes warm.

3. Cytochrome oxidase was assayed in mitochondria treated with digitonin. There is at all times enough cytochrome oxidase to account for the rates of oxidation of succinate and α-ketoglutarate; and enough to account for the relatively slow respiration of the young spadix slices but not for the fastest respiration of the mature spadix.

4. The respiration of spadix slices is found, in contradiction to an earlier report, to be sensitive to malonate.

INTRODUCTION

THE spadix of *Arum* and related plants first caught the interest of plant physiologists over 100 years ago when it was discovered that the spadix becomes warmer than its surroundings as the inflorescence opens. In more recent years a fresh impetus to work on the spadix was given by investigations into the mechanism of carbohydrate breakdown in *Sauromatum* (van Herk, 1937*a*, 1937*b*) and in *Arum* (James and Beevers, 1950). The present work stems from the inability of James and Beevers to detect cytochrome oxidase in the spadix of *Arum*. Following the discovery that mitochondria having Krebs cycle activity could be extracted from *Arum* spadix (Hackett and Simon, 1954), a detailed study was initiated into one step of the Krebs cycle—the oxidation of succinate—since experience with other tissues has been that this oxidation is necessarily mediated through cytochrome oxidase. Only small amounts of cytochrome oxidase could be detected by the conventional procedures in which the mitochondria are challenged with reduced cytochrome *c*, but treatments designed to disintegrate the mitochondria and so allow easier access of this large substrate molecule to its enzyme increased oxidase activity several-fold (Simon, 1957). The most successful technique of disintegration involved treatment with digitonin for 2 hours, but it now seems that this period was too long, for a study of the action of digitonin on

bean mitochondria showed that much higher cytochrome oxidase activities could be realized if the period of treatment were reduced to 30 seconds (Simon, 1958). One purpose of the present paper, then, is to examine with the new technique the cytochrome oxidase activity of mitochondria prepared from the spadix at various stages of development and to relate this activity to the rates of oxidation of two Krebs cycle acids, succinic and α-ketoglutaric. These measurements have a further interest since they may give some insight into the factors governing the rate of respiration of the spadix.

Fresh determinations of spadix respiration rate have been made bearing in mind the possibility that diffusion of oxygen may become a limiting factor when slices are immersed in buffer solution (Yocum and Hackett, 1957) and with particular attention to the sequence of events when the inflorescence opens and the spadix becomes warm.

Finally, the effect of malonate on respiration has been re-examined since James and Beevers reported that the respiration was not sensitive to malonate, a finding which holds the implication that the Krebs cycle does not function in the respiration of this tissue.

MATERIALS AND METHODS

Plant material. Inflorescences of *Arum maculatum* were gathered from local populations and the sterile terminal portion of the spadix excised from them for experiment. The various stages of development of the inflorescence are designated according to the drawings of James and Beevers (1950, Fig. 1) and the following description. While the inflorescence is still immature the spathe remains tightly furled and the three stages α, β, and γ into which its growth is divided are easily recognized with a ruler. This period of growth lasts 2 to 3 weeks but the subsequent development is accomplished more rapidly. In the course of a few hours the spathe unfurls enough to allow sight of the spadix within, and it is at this time that the spadix becomes warmer than its surroundings and emits its characteristic odour. Next day the temperature of the spadix has dropped and its odour gone. The spathe may remain only partly open or it may expand fully but within a couple of days the spathe begins to droop and the spadix to shrivel. According to the drawings of James and Beevers, δ is defined by morphology alone, as the stage at which the spathe is just open enough to show the spadix and in an early series of experiments (Fig. 3) this definition was used. In all the later work, however, plants were only assigned to stage δ if in addition to the morphological criterion they satisfied tests with thermometer and nose. The fully open spadix is designated ε and the final, flaccid stage is ζ.

Respiration of tissue slices was measured manometrically at 25° C. over a 30-minute period. Slices of about 1 mm. thickness were cut from the spadix by hand, washed in distilled water, blotted dry, and then weighed. Five slices (weighing 0·07–0·2 g.) were then added to each manometer flask, 3 flasks being used for each experimental treatment. The slices were either suspended in 2 ml. of 0·05 M. phosphate at pH 7·1 or (for experiments in which it was

desired to avoid the presence of much liquid) placed on filter paper moistened with 0·1 ml. of the phosphate solution.

Preparation of mitochondria. Between 1 and 5 g. spadix tissue was ground in a glass mortar with sand in about 2–10 ml. of a solution containing sucrose (0·5 M.), phosphate (0·1 M., pH 7·1) and versene (0·01 M.). The mixture was passed through muslin and then centrifuged at 1,000 g. for 5 min. yielding a supernatant from which mitochondria were isolated by centrifuging at 12,000 g. for 15 min. The mitochondrial pellet was suspended in a solution of sucrose (0·5 M.) and phosphate (0·1 M.), centrifuged again and finally suspended in a few ml. of the same medium. The whole process was performed in the cold.

For the determinations of cytochrome oxidase (and for the determinations of succinoxidase shown in Fig. 3) the medium used for grinding the spadix and washing, suspending, and assaying the mitochondria was of lower molarity, containing only 0·2 M. sucrose and 0·05 M. phosphate.

Enzyme assays: Cytochrome oxidase was determined by the spectrophotometric method of Simon (1958), the assays being made at room temperature (17–20° C.).

The rate of *oxidation of succinate* (0·02 M.) was measured manometrically at 25° C. for a 30-minute period and in the presence of sucrose (0·5 M.), phosphate (0·1 M.), magnesium (0·001 M.) and adenosinetriphosphate (0·001 M.). For determinations of the rate of oxidation of α-ketoglutarate (0·02 M.) the same medium was used with the addition of diphosphopyridine-nucleotide (3×10^{-4} M.) and coenzyme A (5×10^{-5} M.). Preliminary experiments showed that while the oxidation of α-ketoglutarate was stimulated by these cofactors, additions of diphosphothiamine (10^{-4} M.) and lipoic acid (3×10^{-5} M.) had no influence on mitochondria derived from young spadix material.

Nitrogen was determined by micro-Kjeldahl digestion followed by distillation of the ammonia.

RESULTS

Respiration of tissue slices. When the respiration of slices is measured in the conventional way—with the slices immersed in buffer and a gas phase of air—the rate of respiration is little affected by the age of the spadix being about 1,000μl. O_2/hr./g. at stages α, β, γ, and ε (Fig. 1). However, if the air is replaced with oxygen then the rate is seen to increase with age being doubled by the time γ is reached. The condition at γ, where respiration rate depends on the amount of oxygen in the atmosphere was described by James and Beevers (1950, Table 2) and taken as evidence that the terminal oxidase of respiration had a low oxygen affinity. The elegant experiments of Yocum and Hackett (1957) with *Philodendron* now provide another interpretation of the oxygen effect—namely that when slices are respiring in a liquid phase the diffusion of oxygen may be so slow as to limit respiration rate; the limitation was removed when slices were allowed to respire in a moist atmosphere

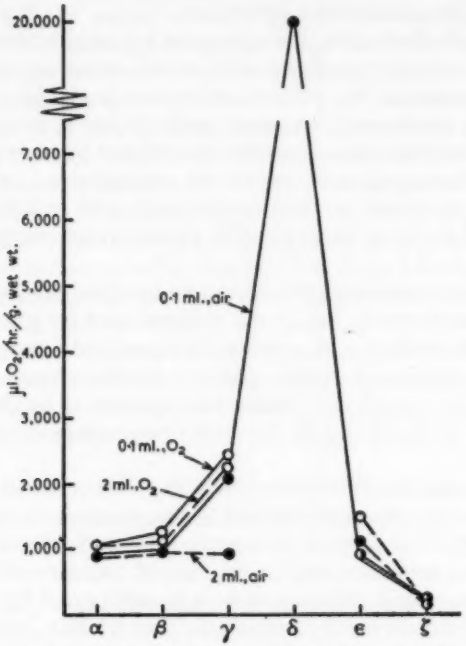

FIG. 1. Respiration of slices of *Arum* spadix at various stages of development. Slices were either immersed in 2 ml. buffer or moistened with 0·1 ml. and were respiring in a gas phase of air or of oxygen.

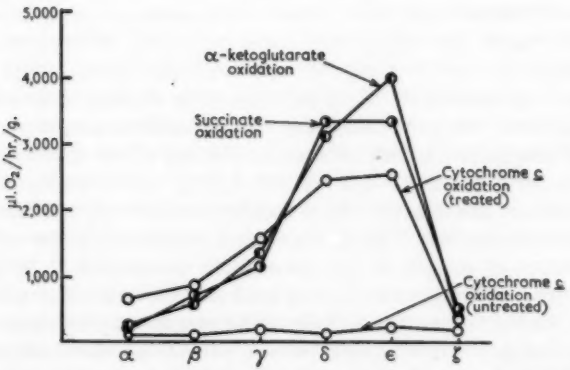

FIG. 2. Oxidase activity of mitochondria prepared from *Arum* spadix material of different ages. Curves are shown for the cytochrome oxidase activity of mitochondria treated with digitonin and for untreated mitochondria.

and respiration was then seen to have a high oxygen affinity. The results of Fig. 1 are readily interpreted on these lines. The respiration of slices immersed in buffer is limited to 1,000μl. O_2/hr./g. when the gas phase is air. At stage γ the rate can be doubled by using oxygen instead of air—or by removing the liquid phase (when the rate in oxygen is little faster than the rate in air).

Experiments with spadix slices at stage δ soon made it clear that very large rates were to be expected and these were measured in the presence of only 0·1 ml. of liquid. Since the spadix is only warm for a few hours, there is bound to be difficulty in finding material at the peak rate of respiration and in fact considerable variation was found (from 4,300 to 21,800μl. O_2/hr./g.). The experimental value plotted in Fig. 1 (20,000μl. O_2/hr./g.) cannot lie far below the maximum rate attained; it is approximately twice the fastest rate observed in *Sauromatum* spadix by van Herk (1937*a*). In the subsequent development of *Arum* spadix, respiration rate falls reaching quite a low level as the spadix loses turgor and begins to wilt.

James and Beevers (1950) concluded from their experiments that malonate did not inhibit the respiration of *Arum* spadix, but there is now so much evidence that the Krebs cycle *can* occur in this tissue (e.g. Hackett and Simon, 1954; James and Elliott, 1955) that it seemed important to re-examine this point. Clear inhibitions were obtained with malonate in 7 out of 8 experiments at stages α, β, and γ. The inhibition was rather slow to develop (Table 1) and it may be that the experiments of James and Beevers were not continued long enough for a sizeable inhibition to appear.

TABLE I

Effect of malonate on respiration of spadix slices suspended in 2 ml. 0·05 M. phosphate, pH 3·5

		% Inhibition due to 0·02 M. malonate			
Stage	Gas phase	1st hour	2nd hour	3rd hour	4th hour
α	air	19	28	47	—
γ	oxygen	35	55	70	80

Mitochondrial oxidase activity. Mitochondria were isolated from spadix material at each stage of development for determinations of enzyme activity. The results (Fig. 2) show clearly that the oxidase activity of the mitochondria obtained from 1 g. spadix tissue increases rapidly as the spadix matures. The rates of oxidation of succinate and α-ketoglutarate are more than ten times faster at stage ϵ than at α.

There appears to be relatively little cytochrome oxidase at any stage when measurements are made on untreated mitochondria, the rate varying between 100 and 200μl. O_2/hr./g. wet wt. Rates of this order have been reported earlier for *Arum* (Simon, 1957) and *Peltandra* (Yocum and Hackett, 1957) and a slightly faster rate—500μl. O_2/hr./g. was recorded for *Symplocarpus* (Hackett, 1957). Treatment of *Arum* mitochondria with digitonin reveals

cytochrome oxidase activities ranging from 700μl. O_2/hr./g. at stage α to 2,500μl. O_2/hr./g. at ϵ.

Taken together, the activities of these three enzymes run a remarkably parallel course as the spadix ages, reaching a peak at ϵ and then declining rapidly when the spadix cells lose their turgor. Two factors could account for the rise in activity on ageing—an increase in the quantity of mitochondrial

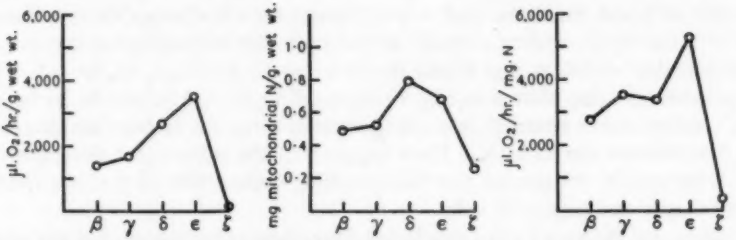

FIG. 3. *Left*: succinoxidase activity of *Arum* mitochondria on a wet wt. basis. *Centre*: quantity of mitochondrial nitrogen brought down in the centrifuge per g. of spadix. *Right*: data of the left-hand curve expressed on a mg. nitrogen basis. All the results are means from two determinations.

material per g. of spadix tissue, and an increase in the specific activity of the mitochondrial enzymes themselves. Measurements of mitochondrial nitrogen (Fig. 3) suggest that both factors are involved. As the spadix ages, the mitochondrial pellet sedimented on centrifuging becomes richer in nitrogen and the activity of the enzymes per mg. nitrogen also increases.

The addition of glucose (0·05 M.) plus hexokinase (1 mg.) or of dinitrophenol (concentrations between 10^{-7} M. and 5×10^{-4} M.) did not increase the rate of oxidation of α-ketoglutarate by more than 10 per cent., suggesting that under the conditions of these experiments the rate was not limited by a requirement for phosphate acceptors.

DISCUSSION

Although the rather feeble cytochrome oxidase activity of mitochondria freshly isolated from *Arum* spadix can be much enhanced by treating them with digitonin, it is necessary to stress that—for several reasons—this procedure still yields an underestimate of cytochrome oxidase activity. It has been shown (Simon, 1958) that prolonged incubation with digitonin reduces oxidase activity and that even in the standard 30-second period of incubation there is some slight decay of activity. A more important factor is that of substrate concentration. Earlier work with *Arum* mitochondria (Simon, 1957) showed the usual hyperbolic relationship between oxidase activity and cytochrome *c* concentration, the activity at infinite concentration (obtained by the extrapolation procedure of Slater, 1949) being 2–3 times higher than that at 20μ M., the concentration used in the present experiments. It is now known that Slater's method itself yields an underestimate since cytochrome *c* is an

inhibitor of the oxidase, its effect becoming more pronounced at higher concentrations (Smith and Conrad, 1956). Finally, it may be noted that cytochrome oxidase was assayed at room temperature, while the other, manometric measurements were made at 25° C.

Accepting then that the cytochrome oxidase figures are too low we may yet inquire how they relate to the rates of carboxylic acid oxidation and to the rate of respiration itself. It seems clear that there is enough cytochrome oxidase to account for the rates at which succinate and α-ketoglutarate are oxidized at all stages of development. It does not necessarily follow that the acids are oxidized in this way and indeed the low activity of succinic cytochrome *c* reductase (about one-tenth that of succinoxidase, Bendall, 1957, and Simon, unpublished) argues against this. It remains for further work to discover whether gentle disruption of the mitochondria allowing the exogenous cytochrome *c* easier access to the enzyme might increase this figure; trials with digitonin have so far been unsuccessful.

Comparison of Figs. 1 and 2 shows that cytochrome *c* oxidase is present in such abundance in the spadix that it *could* account for respiration at most stages of development; but between γ and δ respiration rapidly soars to ten times its previous level while cytochrome oxidase remains unchanged, and here (unless it be that cytochrome oxidase is ten times underestimated) one is driven to conclude that respiration is mediated through some other oxidase. James (1957) has isolated from *Arum* mitochondria a diphosphopyridine nucleotide cytochrome *c* reductase which is autoxidizable and insensitive to cyanide. Bendall and Hill (1956) described a new cytochrome component, b_7, which can be reduced by succinate and malate and is oxidized in air even in the presence of cyanide. This cytochrome occurs in at least ten times the concentration at which *b* components are generally found in plants (see Hartree, 1957). A comparable cytochrome has been reported from mitochondria of skunk-cabbage by Hackett and Haas (1958). It seems likely that cytochrome b_7 mediates oxygen uptake by tissue slices and mitochondria in the presence of cyanide, but it is not yet clear to what extent it functions in the absence of poison (Smith and Chance, 1958).

Two interesting problems are presented by the changes in respiration rate and in mitochondrial activity which occur during development of the spadix. The spasm of respiration at stage δ, a tenfold increase in respiration rate which makes its appearance in the course of a few hours, finds no parallel in the rates of oxidation of carboxylic acids or of cytochrome *c*. The nature of this respiration and of the trigger which sets it off remain as problems for the future. Van Herk (1937*b*) succeeded in obtaining an extract from the male flowers of *Sauromatum* which induced the spasm of respiration when injected into a nearly mature spadix, but nothing comparable is yet known for *Arum*.

The second problem concerns the remarkable increase in oxidase activity with ageing of the mitochondria (Fig. 2). The rates of oxidation of the two acids follow almost parallel curves, increasing more than tenfold between stages α and ε, while cytochrome oxidase shows a slightly smaller increase.

The increase is a consequence of two changes (Fig. 3). There is a small rise in the quantity of mitochondrial nitrogen brought down in the centrifuge, presumably the result of an increase in the number of mitochondria in the tissue or of an increase in their individual size. At the same time the specific activity of succinoxidase increases as the mitochondria age: comparable increases in specific activity have been noted in the germination of seeds of *Pinus* (Stanley, 1957), *Ricinus* (Akazawa and Beevers, 1957) and *Zea* (Hanson, Fisher, and Bils, 1957) and in the development of *Xenopus* embryos (Boell and Weber, 1955) and rat muscle (Shen, 1949, 1955).

While it is possible that the increase in specific activity merely reflects a change in the nature of the particles brought down in the centrifuge such as a reduction in the amount of contaminating protein, the fact that we can now follow comparable trends in tissues as diverse as a spadix, a seedling, and an animal embryo suggests rather that the mitochondria are themselves changing during development. According to this concept the specific activity of the oxidase enzymes increases with age because more of the mitochondrial protein has oxidase activity in the older mitochondria than in the young. The three oxidase enzymes considered here are insoluble and evidence is accumulating that they are located in the opaque membranes seen under the electron microscope in osmium-stained sections of mitochondria (Siekevitz and Watson, 1956; Barrnett and Palade, 1957; Green, 1958; Ziegler, Linnane, Green, Das, and Ris, 1958). Studies of disrupted mitochondria (Hogeboom and Schneider, 1955) show that only about half the mitochondrial nitrogen is located in the membranes, the rest being in the soluble internal phase; so that an increase in the number of oxidase molecules would raise oxidase activity per mg. mitochondrial nitrogen. The new enzyme molecules might be located on the existing membranes, their surfaces becoming more and more crowded with enzyme; or, what is perhaps more likely, there might be a synthesis of extra enzyme-bearing membranes, a proliferation in fact, of the internal cristae or villi. It should be possible to distinguish between these alternatives by electron microscope studies. A preliminary report (Hanson *et al.*, 1957) suggests that there is an increase in the number of cristae in mitochondria of germinating *Zea*. The structure of *Arum* mitochondria is being examined.

This work was aided by grants from the Royal Society. I am much indebted to Mr. J. Martin for his technical assistance.

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The Role of Germination Inhibitors and Oxygen in the Dormancy of the Light-sensitive Seed of *Betula* spp.

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Received 10 October 1958

SUMMARY

Although unchilled, intact seeds of *Betula pubescens* and *B. verrucosa* require light for germination, isolated embryos germinate equally well in both light and darkness.

An aqueous extract of these seeds has germination-inhibitory properties correlated with the presence of a non-fluorescent, single substance. The light requirement of isolated embryos is restored by the inhibitor. When intact seeds are leached with water to remove some inhibitor, it is found that the light requirement is reduced, short days and single light periods then eliciting greater germination than in unleached seeds.

It has been found that scratching, pricking, and cutting the seed coat increases the germination of intact seeds in darkness, and that this is probably due to enhanced oxygen entry. Further, it has been found that germination in short days is increased in oxygen-enriched atmospheres.

It has been found that although the inhibitory effect of the seed coat in intact seeds is partially due to the reduction of the oxygen supply to the embryo, a low oxygen concentration does not prevent germination of isolated embryos. Experimental results suggest that the inhibitor in the seed coat increases the oxygen requirement of the embryo.

INTRODUCTION

IT has been shown that at temperatures below 20° C. germination of unchilled 'seeds' (achenes) of *Betula pubescens* and *B. verrucosa* is under photoperiodic control, greater germination being obtained in long days than in short days, and none in darkness (Black and Wareing, 1955; Vaartaja, 1956). During our investigation, however, it was found that isolated embryos germinate equally well in both light and darkness. This loss of photosensitivity on removal of the seed coat appears to be universal among light-sensitive seeds (e.g. Evenari, 1956; Toole *et al.*, 1956) though the physiological basis of this effect is not understood. This paper reports the results of experimental studies of this phenomenon in birch seed. A preliminary report has been published elsewhere (Black, 1956).

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EXPERIMENTAL

Materials and germination techniques. In these studies unchilled seeds of *B. pubescens* Ehrh. and *B. verrucosa* Ehrh. were used. Seed of *B. pubescens* obtained from open-pollinated trees in southern England and supplied by the British Forestry Commission was used in the earlier experiments. A collection of seed of *B. pubescens* was made in August 1954 from Delamere, Cheshire; this was dried quickly in a greenhouse and stored in darkness at room temperature. Since only a small percentage of this sample contained embryos, it was not suitable for germination studies but was used for extraction purposes. Seed of *B. verrucosa* (1954 harvest) obtained from Messrs. Vilmorin-Andrieux, France, was approximately 35 per cent. fertile and was used for germination studies and extraction work. Seeds were germinated on moist filter paper in petri dishes, which were kept in a thermostatically controlled germination cabinet. In experiments involving irradiation red light within the range 580–700 mμ was used, obtained from red fluorescent tubes in conjunction with red 'Perspex' (R. 400). The intensity at seed level was approximately 1,000 ergs/cm.^{2}/sec.

Extraction techniques. Extracts were obtained by macerating seeds in a blendor in distilled water and allowing the homogenate to stand for 24 hours at 5° C. After filtration, a brown extract was obtained which was sometimes concentrated under reduced pressure (< 32° C.). In several experiments this aqueous extract was fractionated by evaporating to dryness in a freeze-dryer and washing the dried extract with successive solvents.

Chromatographic techniques. For chromatographic studies, extracts were concentrated *in vacuo* to a small volume. Chromatograms were developed with a solvent composed of 99 parts 70 per cent. aqueous isopropanol: 1 part ammonia (S.G. 0·880) until the solvent front was approximately 25 cm. from the starting line. The dried paper was examined in ultra-violet light and the fluorescent zones recorded. For bio-assay the paper was cut into ten equal horizontal strips.

Bio-assay methods. Excised birch embryos and lettuce seed were used for the detection of a germination inhibitor. Birch embryos were obtained from seed kept on moist filter paper in darkness for 20 hours. Dissection was carried out in the light but care was taken not to expose embryos for more than 2 minutes. As an added precaution, the embryos were exposed immediately after dissection, to infra-red radiation (30 mins. at 4,000 ergs/cm.^{2}/sec.), which is known to nullify the effect of promotive radiation. Sections of the dried chromatogram were moistened with equal amounts of distilled water, the amount used being dependent on the size of the strip. For example, to a strip of 12·5 sq. cm. area, 0·5 ml. was added. The embryos were placed on these strips and then in darkness at 18° for 5 days. In addition to this test, which was not very satisfactory because of the large numbers of embryos required, lettuce seed was also used for bio-assay. Light-insensitive lettuce

seed (var. Newmarket) were placed on the strips (30–40 per strip) and kept in darkness for 3 days at 20–21° C.

The extraction and chromatographic separation of a germination inhibitor. Since unchilled, intact seeds of *Betula* require light for germination, whereas isolated embryos germinate in complete darkness, it is evident that the 'seed coat' (pericarp, thin testa, and endosperm) has an inhibitory effect. One possible explanation of this phenomenon is that the seed coat contains a germination inhibitor, and when this is removed the embryo can then germinate. A preliminary experiment established that an extract of 2 g. seed in 10 ml. water markedly inhibits the germination of excised birch embryos and lettuce seed.

This inhibitory action might possibly be brought about by toxic concentrations of substances which are not specific germination inhibitors. Chromatographic studies were therefore undertaken with the aim of separating a specific germination inhibitor from the extract. Aqueous extracts of seeds were found to contain considerable amounts of proteinaceous material and pigment which apparently interfere with the development of the chromatogram. An attempt was therefore made to separate an inhibitory fraction from the remaining extracted material.

Experiment 1. Five grams seed (*B. pubescens*) were extracted with 250 ml. distilled water. After filtration and freeze-drying, the extract was successively washed with 500 ml. each of methanol, chloroform, and water for 2 days at 5° C., with frequent shaking. A series of tests established that extracting in the order methanol, chloroform, and water, succeeded in isolating inhibitory material in the methanol fraction alone and this solvent was used in later experiments for direct extraction. Bio-assay with birch embryos and lettuce seed (Fig. 1) indicates that the inhibition is brought about by non-fluorescent material, highly soluble in methanol and which runs between Rfs. 0·7 and 0·9.

Experiment 2. Because of the small size of the seed it is impracticable to obtain separate amounts of embryos, endosperm, and pericarp large enough for extraction. The material for Expt. 1 was seed containing embryos. However, the collection made at Delamere, Cheshire, was found to contain only approximately 1 per cent. fertile seeds, the remainder of the seeds consisting of pericarp and very poorly developed endosperm; thus, with this sample, extracts can be made mainly of pericarp. Direct methanol extractions were carried out, chromatographed and tested with lettuce seed, and the same inhibitory zone was detected. From this result it appears probable that the inhibitor is located primarily in the pericarp and is not a product of the embryo. Further, it was found that aqueous extracts of both freshly harvested and dried birch leaves contain an inhibitory substance having the same Rf. value and which is possibly identical with that in the seeds.

Experiment 3. Using a technique in which seeds were centrifuged in 50 per cent. aqueous methanol, it was possible to obtain fertile seeds which, because of their density after previously imbibing water, were thrown down during this treatment. The methanol extract of these seeds (4·7 g. fresh wt.)

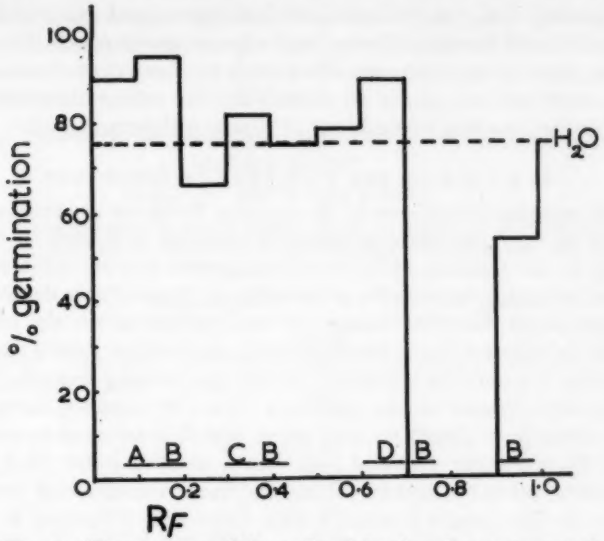

FIG. 1A. Chromatogram of the methanol fraction of an aqueous seed extract, bio-assayed with birch embryos.

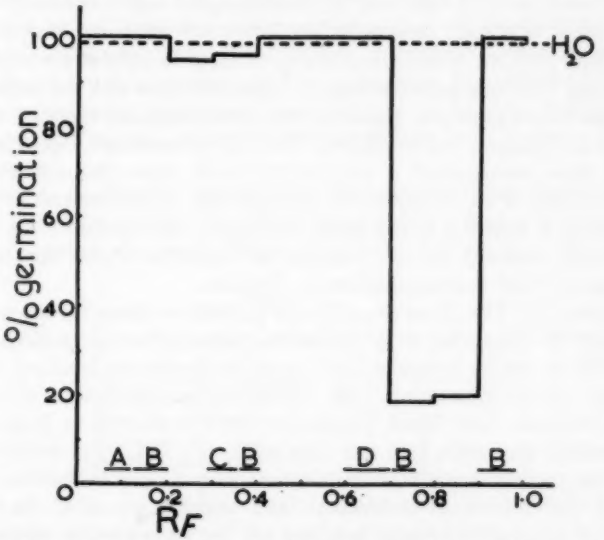

FIG. 1B. The methanol fraction of an aqueous seed extract bio-assayed with lettuce seed.

The following notation is used in recording the U.V. fluorescence: A = pale sky-blue, B = bright sky-blue, C = purple-blue, D = pale purple, E = violet.

was chromatographed and bio-assayed with lettuce seed. No inhibitor other than that located between Rfs 0·7 and 0·9 can be detected. The material remaining after the extraction described above had been carried out was therefore extracted with anhydrous ethyl ether and the extract chromatographed. No inhibitory zone was detectable in bio-assay with lettuce seed.

THE ROLE OF THE SEED COAT IN DOPMANCY

The physiological significance of the inhibitor. Evidence is furnished by the foregoing experiments that a germination inhibitor is present in the seeds, especially in the pericarp, and it may be suggested that the inhibitory effect of the enclosing structures on the germination of the embryo in darkness is due to this substance. On this concept, the embryo *per se* has the capacity to germinate in darkness, but in the presence of the inhibitor light is required for germination. It should be possible, if this is true, to bring about the germination of embryos placed on the inhibitory extract by exposing them to light. Birch embryos were placed on filter paper soaked in an aqueous seed extract obtained by extracting 3 g. seed with 10 ml. distilled water. One group of embryos was kept in red light (1,000 ergs/cm.^{2}/sec.) and the other in darkness. Embryos on filter papers moistened with water were employed as controls, and as a further control intact seeds were used which had been exposed to the same duration of light as that received by the embryos during excision. Fifty embryos were used for each test. The temperature was maintained at 18° C. It was found that light overcomes the inhibitory effect of an aqueous seed extract, 95·7 per cent. germination being obtained in light as opposed to none in darkness. This has been confirmed numerous times and the same effect is observable when embryos, placed on the chromatogram strip in which the inhibitor is localized, are irradiated. That the promotion of germination by light in these experiments is not due to the *in vitro* photo-destruction of inhibitor is shown by experiments in which filter paper, moistened with the seed extract, is exposed to red light. Even after illumination for 4 days, the extract still prevents the germination of embryos which are afterwards placed upon it and then maintained in darkness.

Experiment 2. The above experiments provide evidence for the role of the inhibitor in the dormancy of birch seed but do not give absolute proof of this. The inhibitor can be removed from seeds by prolonged leaching treatment with water. Seeds, placed in a 5 cm. diameter glass vessel, were covered with 5 cm. of washed sterile sand. Distilled water was allowed to drip on to the sand, through the seeds, and was then collected. The water which was collected after passing through the seeds was employed again for further leaching. Leaching was carried out in darkness for 4 weeks at 14–18° C. As a control, seeds were allowed to remain imbibed for the same period on moist filter papers. Chromatography of the leachate using lettuce seed for bio-assay established that some inhibitor had, in fact, been extracted from the seeds. Considerable amounts of inhibitor remained even after this leaching treatment, but leached and unleached seeds showed a markedly different behaviour

in response to various light régimes (Table I). Light is still necessary for germination, however; this may be because some inhibitor is left in the seeds.

TABLE I

The effect of leaching on the percentage germination of seed of birch. (100 seeds used in each test)

(S.L.P. = Single light period)

The variation in the germination under short days can be attributed to small differences in temperature between the two experiments. (See Black and Wareing, 1955 and Vaartaja, 1956.)

	Experiment *a*			Experiment *b*		
Light régime	Leached	Imbibed	Sown dry	Leached	Imbibed	Sown dry
Short days 15° C. .	70·0	20·3	16·8	44·7	1·5	2·1
Long days 15° C. .	90·0	62·5	69·0	95·6	89·7	90·0
Dark 15° C. . .	0	0	0	0	0	0
Dark 21° C. . .	0	0	0	0	0	0
1 hr. S.L.P. 15° C..	25·3	0	2·1	—	—	—
1 hr. S.L.P. 21° C..	39·9	22·5	16·0	—	—	—
7 hr. S.L.P. 15° C..	20·4	5·7	9·0	—	—	—
5 hr. S.L.P. 21° C..	73·7	59·4	55·0	—	—	—
24 hr. S.L.P. 15° C.	20·0	2·1	9·6	—	—	—

Gas exchange phenomena. The foregoing experimental results are consistent with the hypothesis that isolated embryos germinate in darkness because they are removed from the influence of the inhibitor; however, enhanced gas exchange by the embryos is another possible explanation. By making an incision or a scratch in the enclosing structures, gas exchange can be enhanced although the inhibitor is still present. Several experiments have been performed using this technique.

Experiment 1. Seeds were treated in the following ways (approximately 50 for each treatment) and then placed in darkness for 9 days at 18° C.: 1. A single, deep incision was made through the pericarp, testa, and endosperm.

TABLE II

The effect of five treatments on percentage germination in darkness

Pericarp and endosperm slit .	66·6
Pericarp scratched . .	19·3
Pericarp pricked . . .	5·5
Intact seeds . . .	0
Isolated embryos . . .	91·4

2. The pericarp alone was scratched (a single, longitudinal scratch). 3. A single prick was made in the pericarp. The results given in Table II show that although the inhibitor is still present (in the structures enclosing the embryo) germination can be expedited by these operations on the seed coat. Evidently these results cannot be interpreted solely on the basis of an 'inhibitor hypothesis', but can be taken as indicating that a gaseous exchange phenomenon is

operative. A deep incision through the pericarp and endosperm would allow of an easier passage for gases than would a prick or scratch in the pericarp. If the effect is due to better oxygen supply it should be at least possible to increase the germination percentage of cut, scratched, and pricked seeds in darkness still further by submitting them to high oxygen concentrations, and this possibility was tested. The apparatus used to obtain high oxygen concentrations was essentially similar to that described by Denny (1933) with a few minor modifications. Slit, scratched, and pricked seeds were kept in darkness at 18° C. in a range of oxygen concentrations. Isolated embryos and intact seeds were used as controls. The results of this experiment (Table III), show that an oxygen-enriched atmosphere has a marked promotive effect on the germination of those seeds in which the seed coat is treated. This is apparent especially in 50 per cent. oxygen; higher concentrations are obviously toxic, though it is of interest that in several repeats of this experiment light was found to overcome this toxicity.

TABLE III

The influence of oxygen on the percentage germination of slit, scratched, and pricked seeds in darkness

Treatment	Air	50% oxygen	75% oxygen	100% oxygen
Embryos	100	100	69·6	30·4
Intact seeds	0	0	0	0
Pericarp + endosperm slit	70·1	100	80·3	29·6
Pericarp scratched	25·0	100	62·4	24·4
Pericarp pricked	9·4	57·6	59·3	36·1

Although in this experiment and in several repetitions high oxygen concentrations did not induce germination of intact seeds in darkness, it has been found possible to modify the response of intact seeds to light by treating with high concentrations of oxygen. Table IV shows the results of two experiments; these have been confirmed several times. Clearly, in high oxygen tensions, short days are more effective in bringing about the germination of intact seeds than in air.

TABLE IV

The effect of oxygen on the percentage germination of intact seeds in short days (S.D.) 8 hours light/day and long days (L.D.) 20 hours light/day

Oxygen concentration	Experiment *a*			Experiment *b*		
	S.D.	L.D.	Dark	S.D.	L.D.	Dark
Air	15·0	46·3	0	4·1	83·2	0
50%	70·0	84·2	0	45·1	100	0
75%	37·9	65·4	0	10·0	70·0	0

Experiment 2. The results of the preceding experiment demonstrate the beneficial effect of oxygen. It is possible that by scratching or slitting the seed coat increased oxygen is then made available to the embryo, to a level which allows of germination. Further, it might be argued that intact seeds fail to

germinate in darkness because sufficient oxygen to support germination cannot reach the embryo. Whether or not embryos have a requirement for a relatively high oxygen concentration has been investigated. Embryos (25 for each treatment) were placed on moist filter papers in darkness in atmospheres of low oxygen content from 20 per cent. (air) to 0 per cent. (oxygen-free nitrogen).

TABLE V

The percentage germination of isolated embryos in low oxygen concentrations

Oxygen concentration	Continuous light	Darkness
0	76·4	59·3
0·05%	100	100
0·25%	95·0	100
1·0	90·0	95·0
5·0	100	100
10·0	—	100
Air	—	93·7

The results which are given in Table V have been confirmed numerous times and in no case was it found that low concentrations of oxygen prevent the germination of isolated embryos. The seed coat in intact seeds is unlikely to cause as low an internal concentration as, say, 0·5 per cent. when the external oxygen concentration is 20 per cent., still less when it is 75 per cent. (see preceding experiment). Thus, although it seems likely that by interfering with oxygen entry the intact seed coat may prevent the germination of the embryo, it now appears that a low oxygen concentration *alone* is not sufficient to suppress embryo germination, i.e. the seed coat cannot prevent germination by presenting solely a physical obstruction to the inward diffusion of oxygen.

Experiment 3. The experiments above indicate that the inhibitory effect cannot be interpreted in terms of the enclosing structures acting simply as barriers to oxygen entry. Considerable evidence has been obtained which shows that the germination inhibitor is connected directly with the oxygen effect. Seeds were leached in darkness for 4 weeks and the bio-assay of the leachate confirmed that this contained the germination inhibitor. For each test approximately 50 seeds were used and it was found that scratching the seed coat produced a much higher germination percentage of leached than of unleached seed (Table VI). This result cannot be explained on differences in the time of imbibition since a control group of seeds was kept imbibed on moist filter papers. There would also appear to be an enhanced effect of high oxygen concentrations on the germination of intact seeds in darkness when these have been leached. Thus, leaching reduces the oxygen tension necessary to bring about a given percentage germination, indicating that a reduction in the level of inhibitor results in a reduced oxygen requirement by the embryo. Further evidence for this is furnished by the results of experiments in which scratched, leached, and unleached seeds were maintained in darkness in atmospheres composed of low oxgyen concentrations (Table VI). The results are consistent with the above hypothesis, for the germination of scratched

leached seed is reduced only slightly by low concentrations of oxygen in sharp contrast to the response of *unleached* seeds.

TABLE VI

The percentage germination of leached and unleached seeds in different oxygen concentrations.

	Leached	Held imbibed	Imbibed for 2 days
Scratched, in air	60·1	23·8	19·3
Intact, in 50% O_2	10·0	0	0
„ 75% O_2	6·7	0	0
			Imbibed for 1 day
Scratched, 20% O_2	64·3	26·0	21·5
„ 10% O_2	70·6	34·3	20·0
„ 5% O_2	55·3	11·8	9·4

The hypothesis outlined above—that there is a relationship between the oxygen requirement of the embryo and the amount of inhibitor that is present—has been critically evaluated by testing the effect of portions of the (unleached) seed coat on the germination of embryos in very low oxygen concentrations. In this experiment a large part of the enclosing structures was removed so as to bring the embryo into free, direct contact with the atmosphere, each embryo then rested in a boat-shaped portion of the seed coat. After these seeds had been maintained in darkness in low oxygen concentrations, it was found that the germination percentage was very low compared with that of completely isolated embryos (Table VII). This experiment has been performed several times, giving similar results, care being taken to ensure *direct* communication between the embryos and the surrounding atmosphere. These results would appear to justify the conclusion that there is a relationship between the amount of inhibitor present and the oxygen requirement of the embryo.

TABLE VII

The effect of a portion of the seed coat on germination of embryos in low oxygen concentrations

Oxygen concentration	Percentage germination	
	Isolated embryos	Embryos resting on portion of seed coat
0·05	50·0	0
1	90·0	25·0
5	100	50·0
10	100	61·5
20	—	100
50	—	100

The influence of carbon dioxide. In spite of the distinctly promotive effect of

oxygen, it has never been found possible to elicit any germination of intact (unleached) seeds in darkness by treatment with high oxygen concentrations; the reason for this is, at present, obscure. It has been suggested by several workers that carbon dioxide accumulating beneath the seed coat may prevent germination. This is possibly the case in birch seed; if so, high external oxygen concentrations might not be expected to promote germination. Experiments which were performed to test the possibility that carbon dioxide might be one of the natural causes of dormancy of intact seeds in darkness yielded results tending to disprove this. When isolated embryos were maintained in darkness in various concentrations of carbon dioxide, it was found that relatively high concentrations (above 30 per cent.) were required to suppress germination. Furthermore, light does not overcome the inhibitory effect of this gas. High carbon dioxide concentrations (30 per cent.) are required to prevent germination of seeds with the pericarp and endosperm slit, and again light is unable to relieve this inhibition. It is unlikely that carbon dioxide accumulates beneath the seed coat to as high a concentration as 30 per cent., if it all. In addition, the fact that light does not overcome the inhibition by this gas appears to rule out the possibility that carbon dioxide plays any part in the dormancy of the light-requiring birch seed.

DISCUSSION

The factors determining the onset and maintenance of dormancy are only very incompletely understood. However, there is evidence that in some tissues (e.g. dormant buds, potato tuber) inhibitory substances are responsible for the maintenance of dormancy (Hemberg, 1949 *a*, *b*). With respect to seed dormancy, there is no general agreement as to the role of inhibitors in this phenomenon, although their occurrence in seeds is well known (Evenari, 1949) and studies in several species indicate the physiological importance of these substances (Shuck, 1935; Borriss, 1936; El-Shishiny and Thoday, 1953; Wareing and Foda, 1957). For birch seed the evidence would suggest that a germination inhibitor plays a role in the regulation of dormancy. It has been shown that an aqueous extract of these seeds has germination-inhibitory properties which chromatographic studies indicate is brought about by a non-fluorescent substance. It has been shown that the germination of isolated birch embryos is prevented by the inhibitor and that this can be relieved by light. This might be considered as being analogous to the induction of a light-requirement in non-light-requiring lettuce seed by coumarin, a well-known germination inhibitor (Nutile, 1945). The light requirement of intact seeds is markedly reduced when some inhibitor is removed (p. 139); short days are then more effective in promoting germination. The fact that light is still required even after leaching might possibly be attributed to the incomplete removal of the inhibitor (p. 139). Based on these results alone, the hypothesis could be presented that isolated embryos germinate in darkness because no inhibitor is present; with the pericarp, and therefore the inhibitor, still present, light is required for germination. However, the effects of slitting and scratching the

pericarp apparently preclude the acceptance of such an hypothesis, for in these cases the inhibitor is still present but nevertheless germination takes place in darkness. Although not conclusive, there is strong circumstantial evidence indicating that the promotive effects of these treatments can be ascribed to an enhancement of oxygen supply to the embryo as this has been shown to have a stimulating effect on intact seeds and on seeds with the pericarp cut or scratched (p. 140). It would now appear, therefore, that the intact seed coat prevents germination in darkness because it reduces the oxygen supply to the embryo to below a certain critical level required for germination. However, it is apparent that the concept of the seed coat as acting solely as a physical obstruction to oxygen entry must be rejected since it is well proven that isolated embryos are capable of germinating in extremely low concentrations of oxygen (p. 141). It is not intended to imply that the seed coat does not impede oxygen entry, but only that this alone would not prevent germination. Thus, it seems apparent that the embryo has a relatively high oxygen requirement only in the presence of, and induced by, the enclosing structures. The possibility that the occurrence of the inhibitor in the pericarp is connected with the oxygen requirement of the embryo is strengthened by the results of a number of experiments. When some inhibitor is removed by leaching it is found that scratching the pericarp is more effective in promoting germination in darkness. Further, it has been shown that the minimum oxygen tension which allows of the germination of scratched *leached* seeds is considerably lower than that for unleached material. The fact that embryos do not germinate in darkness in low oxygen concentrations if portions of the (unleached) seed coat are present, can be taken as contributory evidence for the direct connexion between the oxygen requirement and the presence of the inhibitor. It would therefore be fully consistent with the experimental results to suggest that the inhibitor actually increases the oxygen requirement of the embryo or, alternatively, that oxygen at certain concentrations is required to overcome the inhibitor. How this is achieved is not clear from the present results. Recent work by Wareing and Foda (1956) on *Xanthium* has yielded substantial proof that an inhibitor in embryos of this species is enzymatically inactivated by oxygen. These workers consider that the beneficial effects of seed-coat removal in dormant seeds is due to an increase in oxygen supply to the embryo, thus bringing about the destruction of the inhibitor. Although there is not, as yet, any experimental evidence for this in birch, the results are explicable on this basis. One of the difficulties encountered with birch seed, however, is the inability to cause germination of intact (unleached) seeds in darkness by treatment with high oxygen concentrations. This can possibly be accounted for by the extremely low permeability of the seed coat to oxygen; but there is no information yet available on this.

Light is necessary to bring about germination when the inhibitor content is sufficiently high and photoperiodic behaviour of the seed can also be considered to depend on the presence of the inhibitor: there is some evidence to support this argument (p. 139). This would appear to be an important result

of the present investigation and might add to our understanding of the photoperiodic control of seed and bud dormancy and even of photoperiodic phenomena in general.

The mechanism of light action is obscure. It is unlikely that light acts in the same way as oxygen, i.e. to destroy the inhibitor, since one would then expect the total quantity of light and not the cycle of light and darkness to be of prime importance (Black and Wareing, 1955). Furthermore, in the present investigation it has been found, in contrast with the results of Redmond and Robinson (1954), that the inhibitor is not destroyed *in vitro* by red light of the intensity that is required to elicit a response in the intact seeds. It is possible that light produces a germination promoter which at a certain threshold concentration can overcome the inhibitor as, for example, in long days.

It has recently been found by us and by Vaartaja (1957) that birch seeds placed on a solution of gibberellic acid (100 gm./l.) will germinate in complete darkness at 20° C. Moreover, it was found earlier (Black and Wareing, 1955) that a period of chilling at 5° C. also removes the light requirement of birch seed. Thus, light, chilling, and gibberellic acid are all effective in overcoming the dormancy of birch seed, and it is possible that all these treatments act by enabling the embryo to overcome the influence of the inhibitor.

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Utilization of Methionine by Mycelial Felts of *Fusarium Culmorum*

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SUMMARY

When the amino-acid L-methionine was used as the sole source of nitrogen in the culture medium it induced appreciable inhibition of growth, respiration, and protein synthesis by mycelial mats of *Fusarium culmorum*. Such inhibitory effects increased with (*a*) lapse of time and (*b*) increase in concentration of methionine in the culture medium.

The results of the experiments presented below support the view that methionine is first oxidatively deaminated before it is taken up by the mycelial mats of the fungus being tested.

INTRODUCTION

In a study of the mechanism of the fungicidal action of mercuric chloride on *Fusarium culmorum* mycelium, the authors (1958) reported that the toxic effect of mercuric chloride as indicated by reduced growth, nitrogen uptake and respiration, and by increased permeability of the plasmatic membranes of the mycelium was intensified by the inclusion of the amino-acid L-methionine in the culture medium containing the toxin. These results were considered as indications of methionine toxicity to growth and metabolic activities of the fungus being tested. Similar observations with respect to methionine toxicity were reported earlier by Robbins and Ma (1945) and Robbins and McVeigh (1946) in case of several fungal species.

The above-mentioned response of the mycelial felts of *F. culmorum* to L-methionine urged the authors to investigate the mode of utilization of this amino-acid as well as its effect when present in culture medium as the sole source of nitrogen on the growth and respiration of mycelial mats of the fungus being tested.

MATERIAL AND METHODS

The fungus used in the present investigation was *Fusarium culmorum* (Smith) Saccardo. It was isolated from damped-off lettuce seedlings and identified by Dr. W. L. Gordon of the University of Manitoba, Canada, to whom the authors are greatly indebted.

The technique used for the preparation of the standard mycelial mats and

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Journ. of Experimental Botany, Vol. 10, No. 28, pp. 146–53, Feb. 1959.

the methods used for the determination of carbon dioxide output by the mycelial mats as well as those used for the estimation of the various nitrogen fractions in the culture media and water extracts of the mycelial mats were those referred to by Tolba and Salama (1957).

A basal medium of the following composition was used throughout: sucrose, 2 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; $FeSO_4.7H_2O$, 0·01 g. in 100 ml.

EXPERIMENTAL RESULTS

In the first experiment of this series 28 samples (mycelial mats) were prepared. Four mats were used for the determination of the initial dryweight and nitrogen content of the mycelium. The remaining samples were suspended over 100 ml. each of sterile water or the sterile experimental solutions, 4 samples being supplied with each of the following: Sterile water; Basal medium; Basal medium+42 mg. potassium nitrate-N; Basal medium+42 mg. L-methionine-N; Basal medium+21 mg. L-methionine-N; Basal medium +14 mg. L-methionine-N.

All culture media so prepared were at pH 5·4–5·5. Such pH value did not reveal any appreciable change throughout the whole experimental period.

The variously treated mycelial mats were incubated at 25° C. for a period of 48 hours during which dry weight, respiration, and nitrogen content of the various culture media and mycelial mats were determined at 24-hourly intervals.

Amino-N utilization. From the results of the analysis of the various culture media for their nitrogen content the amount of amino-nitrogen taken up by the mycelial mats from the various culture media are calculated and the average values are set out in Table I.

It can be seen from Table I that the mycelial mats absorbed large amounts of nitrogen from the various culture media during the first 24 hours of the experiment. On the other hand, the mycelial felts absorbed small amounts of nitrate-N and failed completely to absorb any trace of amino-N from all culture media containing methionine during the second 24 hours of the experiment.

Table I also shows that the mycelial mats absorbed more nitrogen from potassium nitrate- than from methionine-containing media. Varying the concentration of methionine in the culture medium did not materially affect the rate of nitrogen uptake by the mycelial mats from such media. It is to be mentioned, however, that the stoppage of nitrogen uptake from the methionine-containing media cannot be attributed to a depletion of amino-N since all methionine-containing media revealed the presence of varying amounts of methionine-N by the end of the first 24 hours of the experiment. Neither can it be due to any unfavourable pH effects since, as stated earlier, the pH value of all culture media remained, more or less, round the initial value (pH 5·4–5·5) throughout the whole experimental period. Such stoppage

of nitrogen uptake is most probably therefore due to a toxic action of methionine on the mycelial mats of the fungus being tested.

TABLE I

Uptake of nitrate- and amino-N in 48 hours by mycelial felts of F. culmorum from culture media containing potassium nitrate or methionine as source of nitrogen

(*Calculated as mg. nitrogen per mat*)

Source of nitrogen in culture medium	Concentration of nitrogen (mg. N/100 ml. medium)	Nitrogen uptake in 1st 24 hours		2nd 24 hours		48 hours	
		NO_3-N	Amino-N	NO_3-N	Amino-N	NO_3-N	Amino-N
KNO_3	42	9·5	..	2·0	..	11·6	..
L-methionine	42	..	7·0	..	0·0	..	7·0
L-methionine	21	..	7·6	..	0·1	..	7·7
L-methionine	14	..	5·8	..	0·0	..	5·8

The average values of the amounts of protein- and total soluble-nitrogen fractions recovered in the variously treated mycelial mats are contained in Table II, which shows that:

1. The mycelial mats used in the present experiment initially contained almost equal amounts of protein- and total soluble-nitrogen, the values of both of which did not change to any appreciable extent when the mats were suspended over sterile distilled water or the basal medium.

2. Mats growing on all nitrogen-containing culture media absorbed considerable amounts of nitrogen from their media during the first 24 hours of the experiment. All the nitrogen absorbed appeared in the tissue in the form of various nitrogen fractions. Potassium nitrate-nitrogen behaved in a distinctly different manner from methionine-N regarding its distribution among the various nitrogen fractions in the mycelial tissue. Thus, most of the nitrate-N absorbed appeared in the tissue as proteins, a small fraction only was recovered in the form of soluble-N. On the other hand, most of the methionine-N was recovered in the mycelial mats in the form of soluble-N fractions, only a small portion being apparently synthesized into proteins. It is to be observed also that the protein content of the methionine-fed mycelial mats revealed a small but steady increase with the decrease in concentration of methionine in the culture medium.

3. Analysis of the methionine-fed mycelial felts at the end of the second 24 hours of the experiment revealed no change in their content of protein- and total soluble-nitrogen fractions when compared with that observed at the end of the first 24 hours. This is presumably due to the complete stoppage of nitrogen uptake by such mats from their culture media during this time interval. Fungal mats suspended over potassium nitrate-containing media showed small increments in their protein- and soluble-N contents which correspond almost exactly to the small amounts of nitrate-N taken up by the mats under such conditions during the same experimental period.

The above results indicate that the presence of L-methionine in the culture medium as the sole source of nitrogen retarded the synthesis of proteins by the mycelial felts of *F. culmorum* when compared with the rate of protein formation by fungal mats suspended over media containing potassium nitrate as the source of nitrogen.

TABLE II

Analysis of mycelial mats of F. culmorum for their content of protein- and total soluble-nitrogen fractions when suspended for a period of 48 hours over sterile distilled water or culture media containing potassium nitrate or L-methionine or lacking the source of nitrogen

(*Expressed as mg. nitrogen per mat*)

Treatment of the mycelial felts	Nitrogen content after 24 hours		Nitrogen content after 48 hours	
	Total soluble-N	Protein-N	Total soluble-N	Protein-N
Initial	9·4	8·6	9·4	8·6
Suspended over sterile water	9·0	8·3	9·2	8·3
Suspended over basal medium	9·0	8·5	9·1	8·5
Suspended over basal medium +42 mg. potassium nitrate-N	11·4	15·9	12·3	17·7
Suspended over basal medium +42 mg. L-methionine-N	15·4	10·1	15·1	9·9
Suspended over basal medium +21 mg. L-methionine-N	14·3	10·7	14·3	10·3
Suspended over basal medium +14 mg. L-methionine-N	12·2	11·4	12·2	12·2

Analysis of the variously treated mycelial mats for their contents of the individual soluble-nitrogen fractions revealed a marked increase in the amino- and peptide-nitrogen contents during the first 24 hours of the experiment over the initial values with no further increase during the second 24 hours when the mycelial mats were suspended over methionine-containing media.

Calculation of the amounts of the various nitrogen fractions recovered in the different culture media revealed the presence in most of them of very small amounts (not more than 1 mg./100 ml. of medium) of peptide-nitrogen. All media were completely free from any other nitrogen fraction except, of course, the source of nitrogen included in the culture medium.

The facts (*a*) that the utilization of methionine was not accompanied by the liberation of any ammonia in the external medium and (*b*) that methionine utilization led to a marked increase in amino-N content of the mycelial felts may suggest that methionine is taken up as such by the mycelial mats of *F. culmorum*. To get a fair idea about the mode of utilization of L-methionine by the mycelial felts being tested a simple experiment was carried out in the following way. Twelve mycelial mats were prepared. Four of them were suspended over 50 ml. each of sterile distilled water; another 4 were furnished with 50 ml. each of a solution of L-methionine (42 mg. L-methionine-N per

100 ml.) in sterile distilled water, while the third group of 4 mats were fed with 50 ml. each of a sterile culture medium of the following composition: KH_2PO_4, 500 mg.; $MgSO_4.7H_2O$, 250 mg.; $FeSO_4.7H_2O$, 10 mg.; L-methionine-N, 42 mg.; distilled water, 100 ml. All culture flasks were incubated at 25° C. for a period of 48 hours. Analysis of the various culture

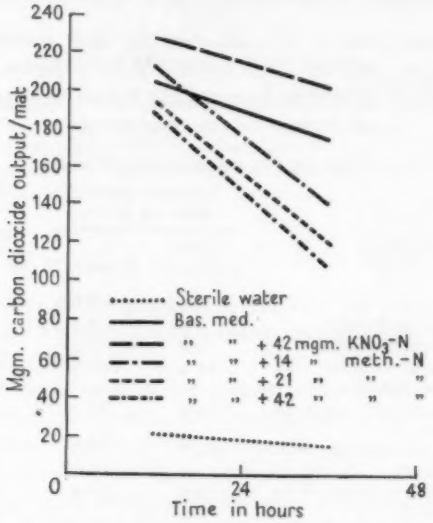

FIG. 1. Carbon dioxide output, over a period of 48 hours, by mycelial mats of *F. culmorum* suspended over sterile water, basal medium, or basal medium to which potassium nitrate or various concentrations of L-methionine were added as source of nitrogen.

media at the end of the experimental period for their contents of the different nitrogen fractions revealed the presence of considerable amounts of ammonia-N in all methionine-containing media. When the latter media were tested for the possible products of methionine utilization by the fungal mats they revealed the presence of α-keto-γ-methylthiolbutyric acid which was detected according to the method given by Sealock (1941) as the 2:4-dinitrophenylhydrazone (m.p. 149°–150° C.).

Carbon dioxide output by the mycelial felts. The average values of the amounts of carbon dioxide given off by the variously treated mycelial mats during the 48 hours of the experiment are plotted graphically in Fig. 1.

Fig. 1 shows that mycelial mats suspended over methionine-containing media respired at a rate much lower than that revealed by mats fed with potassium nitrate-containing medium and even lower than that of mats furnished with the basal medium, i.e. a medium completely lacking any source of nitrogen.

Mycelial dry weight. The average values of the dry weights of the variously treated mycelial mats are contained in Table III. Statistical analysis showed that all mycelial felts fed with the various nutritive media achieved considerable growth during the first 24 hours of the experiment. Those suspended over potassium nitrate-containing medium or the basal medium revealed a smaller increase in their dry weights during the second 24 hours whereas mats furnished with media containing the various concentrations of methionine failed completely to accomplish any growth during the second 24-hour interval.

TABLE III

Dry weights (mg.) of mycelial mats of F. culmorum suspended over sterile water or culture media containing potassium nitrate- or L-methionine-N or lacking the source of nitrogen.

Initial dry weight = 525±22·4

Treatment of the mycelial mats	Dry weight at the end of 1*st* 24 hours	Dry weight at the end of 2*nd* 24 hours
Suspended over sterile water	495± 7·1	480± 0·0
Suspended over basal medium	690± 0·0	735± 7·1
Suspended over basal medium+42 mg. potassium nitrate-N	710±14·1	784±22·6
Suspended over basal medium+42 mg. L-methionine-N	643±18·4	645± 0·0
Suspended over basal medium+21 mg. L-methionine-N	677± 9·9	675±22·4
Suspended over basal medium+14 mg. L-methionine-N	700±14·1	696± 5·7

It can also be seen from Table III that the methionine-containing culture media became more suited for the growth of the mycelial mats being tested as the concentration of methionine in the culture medium was decreased.

DISCUSSION

The results of the present investigation showed that L-methionine-N was taken up in appreciable amounts by mycelial felts of *F. culmorum* within a period of 24 hours. The absorbed nitrogen was built up into proteins and consequently increased the dry weight of the mycelial mats. When fed with methionine-containing media for another 24 hours, the mycelial mats of the fungus being tested failed to accomplish any further growth over that observed by the end of the first 24 hours. This failure was in part at least due to the complete stoppage of nitrogen uptake as well as protein synthesis by the mycelial mats during that time interval. The above facts may be considered as indications of an inhibitory action of methionine on the metabolic activities of the mycelial mats of *F. culmorum*. Toxic effects of methionine were reported earlier by Robbins and Ma (1945) and Robbins and McVeigh (1946) in case

of several fungal species. The inhibitory action of methionine on the metabolic activities of the mycelial felts of the fungus being tested is further substantiated by:

(*a*) the smaller growth of the fungal mats suspended over media containing various concentrations of methionine when compared with that of mats grown on media containing potassium nitrate as the source of nitrogen;

(*b*) the increased growth of the mycelial mats with the decrease in concentration of methionine in the culture medium;

(*c*) the reduced rate of respiration of mats furnished with media containing L-methionine when compared with the rates of carbon dioxide output by mats suspended over media containing potassium nitrate as the source of nitrogen or completely lacking the nitrogen source, and

(*d*) the increased rate of carbon dioxide output by the mycelial felts with the decrease in concentration of methionine in the culture medium.

The mechanism of amino-acid inhibitions in fungi is unknown but, judging from comparable effects in bacterial metabolism where amino-acid inhibitions manifest themselves as media become simplified and synthetic, Foster (1949) suggested that a large excess of a particular amino-acid may competitively block the function of others in the metabolism of the organism. Such blocking may be the reason for the observed inhibition of protein synthesis by mycelial mats of *F. culmorum* suspended over media containing L-methionine as the sole source of nitrogen.

The facts (*a*) that the utilization of methionine was not accompanied by the liberation of any ammonia in the external medium of the fungal mats and (*b*) that methionine utilization led to a marked increase in amino-N content of the mycelial felts may suggest that methionine is taken up as such by the mycelial mats of *F. culmorum*. Alternatively, it may be assumed that methionine is first oxidatively deaminated and the ammonia so formed is rapidly built up into amino-acids. Evidence supporting the view that the latter path is the most probable means of utilization of methionine by the mycelial mats being tested can be obtained from the following two facts:

(*a*) When the fungal mats were grown on a solution of L-methionine in sterile distilled water or a culture medium containing L-methionine and lacking sugar considerable amounts of ammonia-N were recovered in the external media.

(*b*) When the above two media were tested for the possible products of methionine utilization by the mycelial mats, α-keto-γ-methylthiolbutyric acid was isolated from all methionine-containing media as the 2:4-dinitrophenylhydrazone. The conversion of L-methionine to α-keto-γ-methylthiolbutyric acid by a L-amino oxidase enzyme isolated from the liver and kidneys of rats (Blanchard *et al.*, 1944) was reported by Green *et al.* (1944). The enzyme was found also in extracts of *Neurospora* (Horowitz, 1944) and of other fungi (Emerson *et al.*, 1950).

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